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Evaluation of a New Bt Toxin, Cry51Aa2.834_16, for Control of Thrips and Tarnished Plant Bug in Cotton

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To the Graduate Council:

I am submitting herewith a dissertation written by Scott Hester Graham entitled "Evaluation of a New Bt Toxin, Cry51Aa2.834_16, for Control of Thrips and Tarnished Plant Bug in Cotton." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Entomology, Plant Pathology and Nematology.

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Evaluation of a New Bt Toxin, Cry51Aa2.834_16, for Control of Thrips and Tarnished Plant Bug in Cotton

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DEDICATION

I would like to dedicate this work to my wife, Ashlynn. Thank you for your unwavering support during this time in our lives. I would not have been able to do this without your patience and unconditional love.

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ABSTRACT

Field experiments done in 2016 and 2017 in Tennessee evaluated the effects of a Bt toxin, Cry51Aa2.834_16, on the management of thrips (Thysanoptera: Thripidae) and tarnished plant bug (TPB), *Lygus lineolaris* (Palisot De Beauvois), in cotton. For thrips, the Bt trait was as good or better than an insecticide-based approach. The Bt trait reduced numbers of immature TPB and provided partial plant protection from TPB injury. The Bt cotton had greater yields than non-Bt cotton when insecticides were not used. The Bt cotton required fewer insecticide applications to provide adequate plant protection from TPB than the non-Bt cotton. Current treatment thresholds for TPB performed similarly for Bt and non-Bt cotton. Insecticide applications for TPB increased fiber quality, while the Bt trait had minor effects.

Other experiments done in 2016 and 2017 evaluated the behavioral response of thrips and TPB to Bt Cry51Aa2.834_16. Adult thrips avoided Bt cotton in field choice tests and in a test of cotton not treated with insecticides. In a greenhouse choice test more adult thrips and eggs were found on non-Bt cotton than Bt cotton. Similarly, in a field test of Bt and non-Bt cotton not treated with insecticides, 68% of adult thrips were collected on non-Bt cotton. The Bt trait did not affect the distribution of TPB within the canopy of cotton not sprayed with insecticides, although more square and flower injury was caused by TPB in non-Bt cotton. Adult TPB avoided diet containing Bt leaves and excised Bt squares in choice tests with non-Bt squares.

Field experiments were conducted in 2017 and 2018 in Tennessee to determine if an image analysis tool, Canopeo (Oklahoma State University, Stillwater, OK), can be used to supplement current methods to estimate cotton seedling health in small-plot research. Small plot replicated tests analyzed showed a range of cotton seedling health. Cotton seedlings were

visually rated for vigor and thrips injury and above ground biomass samples were also taken. A photograph of the center two rows of each plot was taken using Canopeo. Strong correlations were observed for Canopeo and biomass, Canopeo and vigor, and thrips injury ratings and biomass.

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INTRODUCTION

Cotton

Upland cotton, Gossypium hirsutum L., is among the most important agronomic crops in the mid-south region of the United States. In 2016, there were 494,000 ha of cotton planted in the Mid-South (USDA NASS 2016). Cotton fiber can be used to make linens, paper, and money. Cottonseed produces meal or oil that can be used to feed livestock and humans (National Cottonseed Products Association 2016).

Cotton production ranks in the top three cash crops in the state of Tennessee annually. Cotton is primarily produced in 23 counties in the western region of the state. Historically, Tennessee producers plant an average of 250,000 ha of cotton with yields averaging 650 to 1000 kg of lint per ha. Although there are a limited number of cotton hectares under irrigation, nearly 75% of cotton planted in Tennessee is in no-till or reduced-tillage fields (Main 2013).

Thrips

Biology

Thrips are the most important economic insect pest of seedling cotton in the Mid-South. Thrips belong to the order Thysanoptera which is divided into two suborders – Tublifera and Terebrantia. These suborders are differentiated by oviposition. Species in the suborder Tublifera oviposit eggs on the plant tissue surface, while species in Terebrantia insert eggs within the plant tissue. Thrips are small slender bodied (0.5-5 mm long) insects with or without wings. When fully developed, winged thrips have four long narrow wings with few or no veins. These wings are characteristically fringed with hairs on the edges (Triplehorn 2005). Immature thrips are similarly shaped, but have no wings, are smaller in size, and typically are lighter in color (Layton and Reed 2002, Stewart and Lentz 2010). Thrips have “punch and suck” mouthparts which are used to rupture plant cells and insert their maxillary stylet to extract cellular fluids (Layton and

Reed 2002, Triplehorn 2005). Although adult thrips can fly, thrips are typically wind-blown from field to field (Layton and Reed 2002).

All species of thrips considered to be pests of cotton are in the suborder Terebrantia (Reed et al. 2006). There are five main species of thrips that infest cotton in the United States: the western flower thrips, *Frankliniella occidentalis* (Pergande); flower thrips, *Frankliniella tritici* (Fitch); soybean thrips, *Neohydatothrips variabilis* (Beach); onion thrips, *Thrips tabaci* (Lindeman); and tobacco thrips, *Frankliniella fusca* (Hinds) (Leigh et al. 1996, Reed et al. 2006, Cook et al. 2011). Thrips belonging to the genus *Frankliniella* are recognized as the most important pest species across Mid-South cotton (Freeman et al. 2002, Layton and Reed 2002, Cook et al. 2003, Stewart and Lentz 2010). Within the genus *Frankliniella*, the tobacco thrips is the dominant species found on cotton in the region (Reed and Jackson 2002, Layton and Reed 2002, Cook et al. 2003, Reed et al. 2006, Stewart et al. 2013). Reed et al. (2006) reported that tobacco thrips make up nearly 95% of thrips species found on seedling cotton in the Mid-South.

Thrips can be found on numerous plants species in several plant families. *Frankliniella* spp. have been reported to feed, reproduce, or be collected on up to 49 plant species across the Cotton Belt of the United States. These include plants in the families Asteraceae, Brassicaceae, Convolvulaceae, Fabaceae, Poaceae, Polygonaceae, and Solanaceae (Cook et al. 2011). Thrips typically overwinter as adults on weedy hosts, although larvae have also been observed overwintering, as well as pupae in the soil (Bailey 1938, Chamberlin et al. 1992, Layton and Reed 2002, Stewart and Lentz 2010). Thrips begin reproduction on weedy hosts or winter wheat in the early spring and can complete one generation before cotton emerges (Layton and Reed 2002). Thrips undergo multiple generations per year in the Mid-South (Layton and Reed 2002, Stewart and Lentz 2010). Reed et al. (2006) reported at least some species of the genus

Frankliniella, including the tobacco thrips and flower thrips, can reproduce sexually and parthenogenetically.

The life cycle of Terebrantian thrips begins as an egg laid within the plant tissue and undergoes five stages: egg stage, first larva stage, second larva stage, prepupa, pupa, and imago (MacGill 1927, Layton and Reed 2002). Layton and Reed (2002) reported that under optimal conditions a single female thrips may lay up to 100 eggs during her lifetime. Thrips development varies with species and environmental conditions (Bailey 1938, Lublinkhof and Foster 1977, Lowry et al. 1992, Ullah and Lim 2015). Eggs hatch after 2-23 days. Two plant feeding larval stages follow eclosion. Larvae development lasts 2-13 days. Following the larval stages is a mobile, non-feeding prepual stage. The prepupa drops to the soil after 1-5 days, pupates, and the adult emerges from the soil after 1-10 days. (Hinds 1903, MaGill 1927, Bailey 1938, Lublinkhof and Foster 1977, Lowry et al. 1992). Adult longevity varies by species as well as environmental conditions (Bailey 1938, Lublinkoff and Foster 1977, Ullah and Lim 2015).

Damage to Cotton

Thrips are the key pest of seedling cotton and are consistently among the top 4 pests of cotton overall across the Mid-South. Adults and larvae can injure seedling cotton plants by feeding on the contents of plant epidermal cells, causing a silvery sheen visible along leaf veins and other areas feeding sites (Layton and Reed 2002, Cook et al. 2011, Stewart and Lentz 2010). This silvery appearance occurs when air partially fills damaged cells (Telford and Hopkins 1957, Layton and Reed 2002). As leaves develop, this damage becomes more apparent. The damaged tissue does not form properly causing leaves to be distorted and malformed. Leaf margins also curl upward and inward to the mainstem (Telford and Hopkins 1957, Layton and Reed 2002). This upward cupping is sometimes termed “possum-eared cotton” (Layton and Reed 2002).

Heavy infestations of thrips on cotton seedlings can damage the apical meristem, causing the plant to lose apical dominance. This results in unusual growth and excessive vegetative branching and is commonly called “crazy cotton” (Gaines 1934, Layton and Reed 2002). Studies have shown that seedling cotton injured by thrips can have a negative impact on root growth and development (Roberts and Rechel 1996, Sadras and Wilson 1998). Cotton is most susceptible to thrips injury from emergence until it reaches the three or four true leaf stage (Layton and Reed 2002).

Heavy populations of thrips can lead to stunted growth, delayed fruiting and a reduced stand (Layton and Reed 2002). Thrips injury has been shown to result in a delay of cotton maturity by as many as two weeks (Gaines 1934, Bourland et al. 1992, Parker et al. 1992). However, thrips injury does not always result in significantly delayed maturity (Newsom et al. 1953, Harp and Turner 1976). Thrips damage is typically worse in years with cool weather and drought, which can slow plant growth and increase the amount of time thrips are able to feed on seedlings which can aid in delaying maturity and can lead to increased production costs later in the growing season (Stewart et al. 2013). Seedlings growing in warm favorable conditions are typically less affected by thrips and are less likely to have a delay in maturity (Layton and Reed 2002).

Although the effects of thrips control on seedling cotton can vary from year to year, research has shown an increase of cotton yields when thrips are controlled. Over a 15 year study conducted in the Mid-South, North (2016) reported an average yield increase of 127 kg per ha in cotton treated with an insecticide seed treatment compared cotton with a base fungicide seed treatment only.

Sampling and Threshold

Thrips are primarily controlled with insecticide seed treatments or in-furrow insecticide applications at planting. Occasionally, a foliar insecticide application may also be justified. Scouting thrips in the field is done by sampling 5 to 10 plants throughout several locations in the field. Sample are taken by beating plants onto a white surface, such as a piece of paper or a small white bottomed box. Numbers of adult and immature thrips are then counted and recorded. Depending on the size of the field 50 to 100 plants should be sampled randomly throughout the field. Fields should be scouted at least every 5 days (Layton and Reed 2002). According to the University of Tennessee *Insect Control Recommendations for Field Crops* (Stewart et al. 2016), a foliar insecticide treatment should be considered at the first or second true leaf stage when the emerging leaf show thrips injury and immature thrips are present.

Control Methods

At-planting prophylactic insecticides are typically recommended to manage thrips injury because of the relative quickness that thrips can damage cotton after emergence (Cook et al. 2011). These treatments typically include the use of in-furrow granular or liquid insecticides such as aldicarb or acephate, or seed treatments such as acephate, imidacloprid, or thiamethoxam (Layton and Reed 2002, Catchot et al. 2016, Stewart et al. 2016). The neonicotinoid seed treatments (imidacloprid and thiamethoxam) are widely adopted by cotton growers across the Mid-South (Cook et al. 2011, Stewart et al. 2013). These at-plant insecticides can provide residual control of thrips for 2 to 5 weeks after planting (Ratchford et al. 1989, Graham et al. 1995).

Supplemental foliar insecticide applications may be required when growing conditions are not conducive for seedling growth, when thrips infestations are high, or in the case of control failures with at-planting treatments. Several insecticides are recommended for foliar control of

thrips including acephate, dicrotophos, dimethoate, and spinetoram (Catchot et al. 2016, Stewart et al. 2016). Resistance to neonicotinoid seed treatments is a growing concern across the Cotton Belt. Researchers have reported control issues due to resistance of thiamethoxam and imidacloprid in recent years (Darnell et al. 2015, Darnell et al. 2016, Huseth et al. 2016).

Tarnished Plant Bug

Biology

Tarnished plant bug is in the order Heteroptera and family Miridae (Triplehorn 2005). This family of small, soft-bodied true bugs is characterized by having piercing and sucking mouthparts, four segmented antennae, four segmented proboscis, and lack ocelli. The adult tarnished plant bug is a brown bug with a characteristic yellow-brown Y shaped mark on its scutellum, with reddish-brown antennae (Triplehorn 2005, Leigh et al. 1996). First and second instar nymphs are characterized by a greenish body color, similar to aphids. The early nymphs are distinguished from aphids because they move faster, have reddish tips on their antennae, and lack cornicles. Older nymphs are a green to light brownish color with five characteristic black dots on their dorsum (Leigh et al. 1996).

The tarnished plant bug is a polyphagous insect known to have a wide host plant range. Young (1986) reported up to 385 species or subspecies in 55 plant families that could be host plants for tarnished plant bug across 39 states in the United States (Young 1986). Most the host plants are dicotyledonae ('dicots') particularly in the subclasses Rosidae and Asteridae. The variety of host plants of the tarnished plant bug has allowed the insect to develop a natural tolerance to many of the chemical defensive compounds found in plants (Young 1986). Henbit, *Lamium amplexicaule* L., is an important host for overwintering because it flowers during the winter (Snodgrass et al. 1984). Sour dock, *Rumex crispus* L., and crimson clover, *Trifolium*

incarnatum L., are also good winter hosts and important for reproduction in the spring (Snodgrass et al. 1984). Annual fleabane, *Erigeron annus* L., is thought to be the most important spring host of tarnished plant bug in the Mississippi Delta (Cleveland 1982). These hosts are important because they allow tarnished plant bug populations to build in the spring, and subsequent generations may invade cotton fields. The tarnished plant bug will typically complete one or two generations per year on wild early season weed hosts (Fleischer and Gaylor 1987) before moving into available agronomic crops (Layton 1995, Leigh et al. 1996).

The tarnished plant bug has three distinct life stages: egg, nymph, and adult. The life cycle begins as an adult overwintering in leaf trash (Cleveland 1982). The tarnished plant bug life cycle takes from 22 to 46 days, depending on temperature (Fleischer and Gaylor 1988, Snodgrass et al. 1984). Over the course of the female life cycle, egg production can reach 175 eggs at an average temperature of 27ºC (Ugine 2012). As temperature increases, however, total egg production is reduced, even though the maximum amount of eggs laid per day is the highest at 30ºC. This is because adults live significantly fewer days at 30ºC than they do at temperatures less than 27ºC (Ugine 2012). At 25ºC an average of 7.6 days are needed to incubate tarnished plant bug eggs. At the same temperature, 19.7 days are required to go through the five nymphal instars. Roughly 5 days are required to complete the first instar stage, around 3 days each are required to complete the second, third, and fourth instar stages and roughly 5 days are required to complete the fifth instar stage (Ridgway and Gyrisco 1960). Multiple and overlapping generations occur annually in the southeastern United States (Leigh et al. 1996).

Damage to Cotton

Tarnished plant bug is an important pest of cotton in the Mid-South. It can feed on cotton at any growth stage from emergence to the last maturing bolls (Layton 2000). Feeding typically

occurs on leaf buds and reproductive structures such as flower buds (squares), flowers, and fruit (bolls) (Pack and Tugwell 1976). Early season feeding can result in 'crazy cotton' by causing reductions of plant height and weight, swollen nodes, deformed leaves, and can also lead to a delay in fruiting maturity (Scales and Furr 1968, Hanny et al. 1977). More commonly, tarnished plant bug feeding on squares can cause their abscission and significant yield loss. (Scales and Furr 1968, Scott et al. 1985, Layton 1995). Gutierrez et al. (1997) showed that a single tarnished plant bug can cause the abscission of 0.6 to 2.1 squares per day. As the crop matures, the tarnished plant bug can still cause damage and will also feed on small bolls. Feeding on older bolls (≥ 300 HU) can potentially lead to lint or seed damage but is unlikely to cause boll abscission (Russell et al. 1999).

Tarnished plant bug injects digestive salivary enzymes into plant tissue that breaks the tissues down and assists in the ingestion of nutrients (Pack and Tugwell 1976, Layton 2000). This feeding damages the plant in two ways. The first is mechanical breakdown of the cells at the site of feeding. Secondly, enzymes disrupt plant tissue and is thought to be the more critical aspect of the damage (Layton 1995). Damage from the enzymes injected with saliva are localized and do not appear to be systemic (Layton 2000).

Sampling and Threshold

The tarnished plant bug is primarily controlled with foliar insecticides. Due to this, much research has been done to determine economic thresholds of tarnished plant bug at different growth stages of cotton (Musser et al. 2009). Snodgrass (1993) looked at the density of nymphs in cotton using both drop-cloths and sweep nets. Second and fourth instar nymphs were placed at four locations on cotton plants: leaves, mainstem, terminal, and inside square bracts. Drop-cloths captured more nymphs than the sweep net in every trial, regardless of plant height, release

position, or size of the nymph. This is similar to research done by Young and Tugwell (1976) that found the drop cloth captured 65% of the actual nymph population compared to 16% of the nymph population with the sweep net. Further research by Musser et al. (2007) comparing the drop-cloth and sweep net found similar results to the previous research. The drop-cloth caught more tarnished plant bug nymphs than the sweep net did in both years of the study. The sweep net caught more total tarnished plant bugs in one year than the drop-cloth per sample (25 sweeps or 2 drops) (Musser et al. 2007). The sweep-net also caught more total insects per sample than the drop-cloth (Musser et al. 2007).

When comparing indirect sampling methods, injury to blooms (i.e., dirty-blooms) and the surface of bolls were commonly observed and correlated with tarnished plant bug densities (Musser et al. 2007). The dirty-bloom method was the fastest indirect scouting method, but damage shown is considered to be old damage because it occurs to squares and is not apparent until flowering. Scouting for dirty-squares appeared to be the most effective method of indirect sampling when considering damage found relative to when the damage occurred and the amount of time needed to scout fields. While time of day was not important for total tarnished plant bug or nymph counts for any sampling method, more adults were found later in the day when making whole-plant visual counts (Musser et al. 2007). Musser et al. (2007) compared drop-cloth fabric colors and although adult counts were not significantly different, there was a 22% increase in the number of nymphs found on black drop-cloths compared to white drop-cloths. This has led to the use of a black drop-cloth when sampling for tarnished plant bug because mid-season infestations are often comprised of mainly immature life stages.

Tarnished plant bug population densities and square retention are important factors for lint yield in pre-bloom cotton. Musser et al. (2009b) found that economic losses were minimized

at thresholds of eight tarnished plant bugs per 100 sweeps and 80 to 90% square retention. The percentage of dirty squares (i.e., squares with yellow staining from frass) was a promising predictor of yield reduction in flowering cotton. Plant bug numbers in drop-cloths and sweep-net samples were not as reliable at predicting yield reduction in flowering cotton (Gore 2005). When scouting fields once per week, the economic threshold for tarnished plant bug in blooming cotton should be between 1.6 to 2.6 plant bugs per 1.5 m of row (Musser et al. 2009). A threshold of 5-10% dirty squares is equivalent to the threshold given by Catchot (2016) of three tarnished plant bugs per 1.5 m of row (= 3 per drop cloth) and provides a similar economic return in blooming cotton (Gore et al. 2007).

Control Methods

Chemical insecticides are currently the most widely used method of control for tarnished plant bug in cotton in the Mid-South. Several classes of insecticides are needed to control this pest. Due to growing resistance to organophosphates and pyrethroids, neonicotinoid insecticides are becoming more prevalent for control in both pre-bloom and blooming cotton. Imidacloprid (e.g., Admire Pro, Bayer CropScience, Raleigh, NC) and thiamethoxam (e.g., Centric, Syngenta Crop Protection, Greensboro, NC) are the most commonly used neonicotinoids for control of tarnished plant bug (Gore et al. 2007). The insect growth regulator novaluron (e.g., Diamond® 0.83EC, ADAMA USA, Raleigh, NC) and flonicamid (e.g., Carbine, FMC Corporation, Philadelphia, PA) represent newer classes of insecticides used for tarnished plant bug control in cotton (Gore et al. 2007). Appropriate timing of both initial applications and subsequent applications is key to satisfactory control of tarnished plant bug. To reduce selection for insecticide resistance, the *Insect Control Recommendations for Field Crops* for Tennessee (PB 1768, Stewart and McClure 2017) includes an insecticide rotation strategy for tarnished plant

bug management. The use of neonicotinoids or flonicamid is recommended prior to flowering after which organophosphates, carbamates, pyrethroid and novaluron are recommended either alone or in a tank mix. Fields should be scouted twice a week to ensure populations are controlled as soon as threshold is reached (Gore et al. 2007).

With the declining efficacy of most insecticides against tarnished plant bug, a multi-tactical approach is needed to obtain economical control. Adams (2012) showed that early planting dates combined with an early maturing cotton variety can significantly reduce the number of insecticide applications needed to control tarnished plant bug throughout the growing season. Reducing plant height and opening the plant canopy with plant growth regulators, such as mepiquat chloride, can potentially increase the effectiveness of insecticides needed late in the growing season (Graham 1985). Applying selective herbicides to areas such as turn rows, ditches, and roadsides for control of early season hosts can effectively help to reduce the cost of control later in the growing season (Snodgrass 2003, Snodgrass et al. 2006, Gore et al. 2010). Nectariless varieties have significantly fewer nymphs when compared to nectaried varieties (Schuster et al. 1976, Bailey et al. 1984). This could be due to reduced tarnished plant bug populations as well as reduced fecundity of plant bug females on nectariless varieties (Schuster et al. 1976). The combination of early planting and nectariless varieties shows resistance to plant bugs (Milam et al. 1985), although nectariless cotton varieties are not typically grown commercially.

Resistance

Bt cotton, combined with the eradication of the boll weevil, *Anthonomus grandis grandis* Boheman, has caused the tarnished plant bug to go from a secondary pest to a primary pest, because applications targeted at these pests, which provided management of the tarnished plant

bug, are no longer required (Musser et al. 2009). Applications made for tarnished plant bug or other pests have selected for resistance. Tarnished plant bug resistance to methyl parathion in the Mississippi River Delta was first documented in a 1979 study by Cleveland and Furr (1979). Resistance to dimethoate was reported in the Mississippi Delta by Snodgrass and Scott (1988), but there was little tolerance to acephate found. Resistance to pyrethroids, organophosphates, and cyclodiene insecticides in the Mississippi Delta were reported in 1996 (Snodgrass 1996). Widespread resistance to pyrethroid insecticides has been reported in the Mississippi River Delta regions of Mississippi, Arkansas, and Louisiana (Pankey et al. 1996, Hollingsworth et al. 1997, Snodgrass and Scott 2000, Snodgrass 2006) and is also well established in Tennessee. Acephate resistance was documented in one county of the Mississippi Delta in 2005 (Snodgrass 2006), and was widespread across the region by 2006 (Snodgrass and Gore 2007a, Snodgrass et al. 2009). However, acephate remains one of the most commonly used insecticides for the control of plant bug.

Bacillus thuringiensis (Bt) Cotton

Bacillus thuringiensis Berliner (Bt) has been known to have insecticidal properties for over a century. It was first isolated by a Japanese scientist in 1901 (Ishiwata 1901). Ernst Berliner was given credit for officially documenting the bacterium nearly 10 years later. The first Bt product, Thuricide, was commercialized in 1957 (Beegle and Yamamoto 1992). Several other foliar Bt products were produced in the following decades, but were not widely adopted due to how easily they were degraded in the light. They also tended to be less popular than synthetic pyrethroids because the Bt must be ingested by the target organism to affect it (Beegle and Yamatoto 1992). The utility of Bt toxins increased substantially beginning in 1996, when the first transgenic crops which expressed Bt toxins became commercially available (Perlak et al.

2001). These varieties provided excellent control of the heliothine complex. The heliothine complex consists of two insect caterpillars that feed on fruiting structures of cotton, the tobacco budworm and bollworm (Siebert et al. 2008).

The widespread adoption of Bt cotton caused a shift in the key insect pest of cotton from the heliothine complex to the tarnished plant bug (Musser et al. 2009). While many Bt proteins have been shown to have excellent insecticidal activity against lepidopteran, coleopteran, and dipteran insects (Schnepf et al. 1998, Van Frankenhuyzen 2009), few have shown adequate activity against hemipteran insects (Walters and English 1995, Porcar et al. 2009). However, Baum et al. (2012) reported a Bt protein with insecticidal activity against both *Lygus hesperus* and *Lygus lineolaris.* This protein was reported as Cry51Aa2. Gowan et al. (2016) reported the introduction of Cry51Aa2 into a cotton varieties. Four transgenic events were selected for field trials. These transgenic events showed 7- to 19-fold fewer *L. lineolaris* compared to the untreated check in field bioassays. One transgenic event in particular, GH_A710504 (expressing Cry51Aa2.834_16) showed levels of efficacy that should provide substantial control of *Lygus* bugs in cotton. Thus, GH_A710504 was chosen to be further studied as a *Lygus* control product designated as MON88702.

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CHAPTER I

Field Study Investigating Cry51Aa2.834_16 in Cotton for Control of Thrips (Thysanoptera: Thripidae) and Tarnished Plant Bugs (Hemiptera: Miridae)

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Abstract

Field experiments were conducted in 2016 and 2017 in Tennessee to evaluate the effects of a novel Bt-transgenic toxin, Bt Cry51Aa2.834_16, on thrips (Thysanoptera: Thripidae) and tarnished plant bug (TPB), *Lygus lineolaris* (Palisot De Beauvois), in cotton. Protection from thrips injury with the Bt trait was as good or better than an insecticide-based approach. The use of the Bt trait resulted in reduced numbers of immature TPB, particularly large nymphs, and partial protection from plant bug injury. Cotton that expressed Bt Cry51Aa2.834_16 had greater yields than the non-Bt isoline when insecticides were not used. Although Bt Cry51Aa2.834_16 reduced the need for insecticide applications, foliar-applied insecticide applications were needed to provide adequate plant protection from TPB. The current recommended treatment thresholds for TPB performed similarly well for Bt Cry51Aa2.834_16 and non-Bt isolines. Insecticide applications for TPB increased fiber quality, while Bt Cry51Aa2.834_16 had minor effects. The Bt-transgenic toxin Cry51Aa2.834_16 is expected to reduce the need for insecticide applications targeting thrips and TPB and could be a valuable addition to an overall insect management program in cotton.

Keywords: Thrips, Tarnished Plant Bug, Bt cotton, Cry51Aa2.834_16, Insecticides

Introduction

Cotton, *Gossypium hirsutum* L., is one of the top three cash crops grown in the Mid-South (USDA NASS 2017). Over the last ten years, thrips (Thysanoptera: Thripidae) have ranked among the top three insect pests in cotton based on costs to producers (Cook 2018).

Among the thrips species that attack seedling cotton, tobacco thrips, *Frankliniella fusca* (Hinds), are the most common species found in the Mid-South and Southeast (Reed and Jackson 2002, Layton and Reed 2002, Reed et al. 2006, Stewart et al. 2013). Thrips injury can lead to stunted growth, delayed maturity, reduced stands, and yield loss (Layton and Reed 2002, Stewart and Lentz 2010). Currently, thrips are primarily managed using prophylactic, at-planting insecticide applications, such as seed or in-furrow treatments. The neonicotinoid seed treatments, namely imidacloprid and thiamethoxam, are widely used by cotton growers (Cook et al. 2011) and can provide protection for several weeks after planting (Graham et al. 1995), but there have been growing concerns about thrips resistance to neonicotinoids. Documented control issues in the mid-south (Darnell et al. 2015, 2016) and the southeast (Huseth et al. 2016) have led to the need for new ways to combat this pest.

The tarnished plant bug (TPB), *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae), typically ranks as the most important insect pest of cotton in the Mid-South. (Cook 2018). This pest can feed on plants at any growth stage, but most economic damage is done from the onset of squaring (flower buds) through the blooming period (Layton 2000). TPB can cause the abscission of squares and bolls, resulting in substantial yield loss (Scales and Furr 1968, Scott et al. 1985, Layton 1995, Russell 1999). Foliar-applied insecticide applications are often used to manage infestations of TPB. Insecticides are often rotated and/or tank mixed to help maintain effective control (Catchot et al. 2014), but the TPB has developed resistance to several insecticides (Cleveland and Furr 1979, Snodgrass and Scott 1988, Snodgrass 1996, Snodgrass and Scott 2000, Snodgrass 2006, Snodgrass et al. 2009, Parys et al. 2017). This has made management of TPB increasingly difficult, leading to the need for new methods to manage this pest.

The use of transgenic cotton expressing *Bacillus thuringiensis* (Bt) has been widely adopted (Fernandez-Cornejo et al. 2014). These cotton varieties express toxins targeting the control of lepidopteran pests, including corn earworm, *Helicoverpa zea* (Boddie), and tobacco budworm, *Heliothis virescens* (F.) (Siebert et al. 2008). The adoption of these Bt varieties caused a shift from lepidopteran pests to other pests, especially the TPB in the Mid-South. (Musser et al. 2009). Current Bt crops do not provide control of hemipteran insects like the TPB. However, Monsanto Company (St. Louis, MO) has developed a new Bt protein, Cry51Aa2.834_16, referred to as Bt Cry51Aa2 from here on. This toxin has insecticidal activity against several insect pests in the family Miridae, including *Pseudatomoscelis seriatus* (Reuter), *Lygus hesperus* (Knight) and TPB (Baum et al. 2012). In 2016, Gowda et al. reported the incorporation of this Bt trait into cotton. In addition to insecticidal activity against mirids, this protein also has activity against at least some thrips (Bachman et al. 2017). Thus, the objective of this study was to evaluate the efficacy and potential impact of this novel Bt toxin on the management of thrips and TPB, both being important pests of cotton in the Mid-South.

Materials and Methods

Plot Establishment

Experiments were conducted in 2016 and 2017 at the West Tennessee Research and Education Center (WTREC) in Jackson, TN and the Research and Education Center at Milan (MREC) in Milan, TN. Prior to planting, seed were treated with a fungicide seed treatment (Trilex Advanced; Bayer Crop Science, Raleigh, NC) at a standard recommended rate. Trials in 2016 were planted on 5 May at WTREC and on 9 May at MREC. In 2017, trials were planted on 9 May at WTREC and on 10 May at MREC. A seeding rate of ~13.2 seed per m was used in all cases and all tests were planted into no-till production fields. Plots at WTREC were irrigated

with a lateral irrigation system and plots at both locations were fertilized and managed for weeds according to University of Tennessee Extension recommendations (Steckel et al., 2016, Savoy and Joines 1996).

Treatment Factors

Treatments were arranged in a split-split-plot experimental design within a randomized complete block with four replications. Main plots were 16 rows wide (0.97 m centers at WTREC and 1.02 m at MREC) and 10.7 m long and consisted of three foliar-applied insecticide regimens for TPB including 1) no applications, 2) applications based on current recommended thresholds, and 3) automatic/aggressive applications made weekly. Sub-plots were 8-rows of either Bt Cry51Aa2 or non-Bt. Seed for each was provided by Monsanto Company (St. Louis, MO) and were near-isogenic lines of the same variety (DP 393). Sub-subplots were 4-rows wide and consisted of fungicide-only treated seed or seed treated with a fungicide and an insecticide seed treatment (IST). Aeris (Bayer CropScience, Raleigh, NC), a combination of imidacloprid and thiodicarb, was used as the IST and applied at a standard rate of 0.75 mg ai/seed. Cotton treated with Aeris was also treated with 271.81 g ai/ha of acephate (Orthene 97S, Amvac Chemical Company, Walnut Creek, CA) at the first true-leaf stage.

The treatment threshold and foliar insecticides used to control TPB were those recommended in the *Insect Control Recommendations for Field Crops* for Tennessee (Stewart et al. 2017). For plots treated according to threshold, the decision to treat was made independently for each Bt by IST factor combination based on average pest density in the four sub-subplots. During the squaring period, the threshold was defined as 8 bugs per 100 sweeps or when square retention fell below 80%. At the initiation of bloom, a threshold level of 3 bugs per 1.52 row m, based on drop cloth sampling was used to make treatment decisions (see below). Automatic

applications of insecticides for TPB in the aggressive treatment regimen were made weekly beginning the first week squares were observed. The insecticides used varied depending upon the time of season and other hemipteran pest species such as clouded plant bug, *Neurocolpus nubilus* (Say)(Hemiptera: Miridae), and stink bug (Hemiptera: Pentatomidae) infestation levels. Foliar tank-mixed applications of imidacloprid (Admire Pro, Bayer Crop Science, Raleigh, NC) at a rate of 68.4 g ai per ha and thiamethoxam (Centric 40WG, Syngenta Crop Protection, Inc., Greensboro, NC) at a rate of 35.0 g ai per ha were primarily used control TPBs prior to bloom. Around the first week of bloom, novaluron (Diamond 0.83EC, ADAMA USA, Raleigh, NC) was added to the tank mixture at a rate of 43.6 g ai per ha. Once blooming began, we used treatments of sulfoxaflor (Transform WG, Dow AgroSciences, Indianapolis, IN) at a rate of 52.5 g ai per ha or tank-mixtures of acephate (Orthene 97, Valent USA, Walnut Creek, CA) and bifenthrin (Brigade 2EC, FMC Corporation, Princeton, NJ) at 727.9 g and 87.5 g of ai per ha, respectively. Since tested lines did not express a Bt toxin for control of lepidopteran pests, applications of chlorantraniliprole (Prevathon, DuPont Crop Protection, Newark, DE) were made at 75.3 g ai per ha to minimize any potential effects of lepidopteran pests while having minimal effects on TPB populations.

Thrips Sampling

Cotton was sampled at the 1.5 and 3.5 leaf stage to estimate the density of thrips. Five plants were sampled from each sub-subplot. Samples at the 1.5 leaf stage were collected 5-6 days after the foliar application of acephate was made to cotton with an IST. Plants were cut at the ground level and placed in 16 oz jars containing 70% ethyl alcohol. Each plant was taken out of the jar and rinsed with 70% ethyl alcohol over a glass container topped with a sieve (150 μm) to collect the thrips. The jar was then rinsed with 70% ethyl alcohol over the sieve to collect any

remaining thrips left inside. The sieve was then rinsed with 70% ethyl alcohol into a gridded 100 mm x 15 mm petri dish and the thrips counted underneath a microscope. Thrips were counted and categorized as either adult or immature. Adult thrips were classified as either tobacco thrips, soybean thrips, *Neohydatothrips variabilis* (Beach), or other thrips. The same procedure was used for collections at the 3.5 leaf stage, except plants were placed in a 32 oz plastic bag, without alcohol, in order to collect plant biomass data prior to washing thrips from the plants. The fresh weight of each sample, minus the weight of the bag, was recorded and the samples were placed in a refrigerator until thrips were counted within the next 24 h.

Whole-plot, visual ratings of thrips injury and vigor were taken at the same growth stages. Vigor ratings were made on a 0 – 5 basis, with 0 representing no living plants in the plot and 5 representing maximum vigor. Thrips ratings were also on a 0 – 5 scale, with 0 representing no injury to any plant in the plot and 5 no living plants in the plot. Also, total squares per 1-m row were counted in each sub-sub plot at the first week of squaring to help assess thrips injury effect on cotton maturity.

Tarnished Plant Bug Sampling

Methods of estimating TPB infestations and injury varied depending upon the growth stage of the cotton. Weekly sampling of TPB began at first-square. Prior to flowering, samples were taken from the center two rows with a 38.1-cm diameter sweep net by taking 25 sweeps in each sub-subplot. Square retention was monitored by examining the first position fruiting sites on the top two nodes, excluding the terminal node, of plants until 25 sites were examined in each sub-subplot. The number of retained squares was recorded. A square was considered missing if it abscised when touched or the bracts were flared. Beginning at the third week of squaring and through much of the blooming period, TPB densities were estimated using a black drop cloth.

Samples were taken by laying the cloth between two cotton rows near the center of the plot and vigorously shaking all of the plants from each row. One sample resulted in 1.52 m of row being sampled. TPB nymphs and adults were recorded separately. TPB nymphs were visually separated based on size as either small (1^{st} or 2^{nd} instars) or large (3^{rd}, 4^{th}, and 5^{th} instars). Sampling was terminated when cotton reached five nodes above white flower plus 350 heat units (DD60s°F). Numbers of clouded plant bugs and stink bugs were also recorded at each sample date, as these pests could potentially be impacted by this technology and have influence on fruit injury and yield.

To determine overall fruit retention during the squaring period, a total of ten plants per sub-sub plot was sampled from the middle two rows to estimate percent retention of first position fruiting structures at the first week of bloom. Plants were examined at each fruiting branch and the number of present first position fruit per node were recorded.

Yield

In all trials, the center two rows of each sub-subplot were harvested and seed cotton weights were recorded. At the WTREC location, seed cotton samples (≈1.0 kg) from each sub-subplot were collected in 15.2 cm x 9.5 cm x 27.3 cm paper sacks to be sampled in a table-top gin to determine lint turnout and fiber quality. Because this Bt Cry51Aa2 is under USDA regulation, great care was made to keep collected seed cotton samples in a contained and restricted environment until ginned, and all seed from the ginning process were devitalized before disposal.

Data Analysis

Sample date was not included in the model because insecticides were applied to each treatment and in each test independently. Each treatment received insecticide applications a

different number of times and at different timings during the season, making interpretation of data difficult. Therefore, numbers of thrips, thrips injury ratings, plant vigor, pre-bloom square retention, TPB numbers in sweep nets, and tarnished plant numbers on drop cloths were analyzed across all sample dates to show the overall impact of each management strategy on those variables. All data were analyzed using a general linear mixed model of analysis of variance PROC GLIMMIX of SAS (Version 9.4, SAS Institute, Cary, NC). Insecticide seed treatment (IST), Bt Cry51Aa2 (trait) and spray regimen (spray) were designated as fixed effects. Year, location, year by location, and replication nested within year by location were designated as random effects to allow inferences to be made over a range of environments (Carmer et al. 1989, Blouin et al. 2011). Degrees of freedom were estimated using the Kenward-Roger method (Kenward and Roger 2009). Means were estimated using LSMEANS and separated based on Fisher's protected least significant difference (α=0.05). Three-way interactions were not included in the model for thrips related data because foliar applications for TPB had not been initiated at this time.

Results

Thrips

A significant interaction of insecticide seed treatment (IST) by trait on the average total number of adult and immature thrips (F=70.16; df=1, 364; P<0.001) was observed. The majority of thrips found were immatures (78.4%). Based on adults, tobacco thrips composed nearly 71% of the population. The most thrips, 78.1±4.95/5 plants (mean ± standard error) were found on non-Bt cotton that did not have an IST (Fig. 1, Supp. Table S1). No significant difference between total thrips on Bt Cry51Aa2 cotton without an IST and non-Bt cotton treated with an IST and a foliar insecticide application was observed. The fewest number of thrips (7.8±0.66/5

plants) was found on Bt Cry51Aa2 cotton treated with an IST and a foliar insecticide application (Fig. 1, Supp. Table S1).

For thrips injury, an interaction of IST by trait (F=444.1; df=1, 356; P<0.001) was observed. Non-Bt cotton without an IST had more thrips injury (3.02±0.08) than all other treatments (Fig. 1, Supp. Table S1). Bt Cry51Aa2 cotton without an IST had less thrips injury than non-Bt cotton treated with an IST and a foliar insecticide application, and Bt Cry51Aa2 cotton treated with an IST and a foliar application of acephate had the least injury (0.64±0.03) (Fig. 1, Supp. Table S1).

There was no effect of trait on average above ground biomass of seedlings (F=0.04; df=1, 146.8; P=0.843). However, an effect of IST was observed (F=43.89; df=1, 146.8; P<0.001) where cotton treated with an IST had greater biomass than cotton without an IST (Fig. 1, Supp. Table S1). No interaction between IST and trait was found, although it did approach significance (F=3.78; df=1, 146.8; P=0.054). There was an interaction of IST by trait on whole plot vigor ratings (F=35.80; df=1, 85; P<0.001). Similar to biomass, cotton treated with an IST had higher vigor ratings, regardless of trait. However, the Bt Cry51Aa2 cotton without an IST had more vigor (4.18±0.18) than non-Bt cotton that did not have an IST (2.90±0.10) (Fig. 1, Supp. Table S1).

The use of an insecticide seed treatment (F=1.15; df=1, 173; P=0.284) did not affect the total number of squares present during the first week of squaring. However, there was an effect of trait (F=5.17; df=1, 173; P=0.024), with more squares in Bt Cry51Aa2 cotton (26.29±1.44) than in non-Bt cotton (24.20±1.23). No interaction of IST by trait was found (F=0.15; df=1, 173; P=0.067).

Tarnished Plant Bug

Based on current threshold recommendations for TPB, 1 to 7 insecticide applications were needed to manage TPB depending on the year and test location (Fig. 2, Supp. Table S2). In both years, more insecticide applications were needed at the Jackson location than in Milan, and more applications were required on non-Bt cotton than the Bt Cry51Aa2 cotton. On average across the four trials, the Bt Cr51Aa2 cotton required 1.25 fewer insecticide applications for TPB than non-Bt cotton when treated according to threshold recommendations.

Unless indicated, three-way interactions were not significant (P>0.05) and are not discussed. No two-way interactions were found for IST by trait (F=0.00; df=1, 825.7; P=0.959), IST by spray (F=1.26; df=2, 825.7; P=0.284), or trait by spray (F=1.67; df=2, 827.7; P=0.189) on the average number of TPB adults found prior to bloom. Insecticide seed treatment did not have significant effect on the average number of TPB adults found on cotton prior to bloom (F=0.49; df=1, 825.7; P=0.483), but there was a significant effect of trait (F=14.94; df=1, 825.7; P<0.001) and of spray regimen (F=21.54; df=2, 825.7; P<0.001). About 23% more adults were found on non-Bt cotton (2.68±0.13) than on Bt Cry51Aa2 cotton (2.07±0.11). Also, there were significantly more adult TPB found in cotton that was not sprayed with insecticides (3.00±0.18) compared with the other treatment regimens and more adult TPB were found in cotton managed using the threshold approach (2.39±0.14) compared with the more aggressive treatment regimen (1.73±0.11).

Overall, square retention stayed above 80%, regardless of the treatment factors (Fig. 6). However, a three-way interaction of IST by trait by spray regimen on average square retention was observed (F=3.53; df=2, 824.4; P=0.029). Square retention in Bt Cry51Aa2 cotton stayed above the average retention of the trial treatments (91.04±0.86%), regardless of IST or spray regimen (Fig. 6). Square retention was greater in the Bt Cry51Aa2 cotton than the non-Bt when

no insecticide applications were made. Unless the non-Bt cotton had an IST and was sprayed automatically, its retention was below the average retention of the trial treatments (Fig. 6). At first bloom, first position square retention was mapped to determine the overall retention during the squaring period of the season. There was no interaction of IST by trait (F=0.01; df=1, 165; P=0.918) or IST by spray regimen (F=2.26; df=2, 165; P=0.108). There was an interaction of trait by spray regimen (F=3.33; df=2, 165; P=0.039). In non-Bt cotton, there was significantly higher square retention in the threshold approach (81.77±1.25%) compared to unsprayed (74.97±2.04%). In the Bt Cry51Aa2 cotton, there was no difference between the threshold approach (85.08±0.76%) and cotton unsprayed for TPB (82.91±1.05%). Significantly higher square retention was observed in Bt Cry51Aa2 cotton sprayed aggressively with insecticides (90.39±0.86%) compared to all other treatments, followed by non-Bt cotton sprayed aggressively (87.14±0.98%). Non-Bt cotton that was not treated for TPB had the lowest overall square retention.

The majority of TPB observed from samples taken during the blooming period were nymphs (92.4%). There was an interaction of IST by spray regimen on the average number of TPB nymphs found in drop cloth samples (F=7.58; df=2, 847.1; P<0.001). Significantly more nymphs were found in cotton not treated with foliar insecticides for TPB than all other treatments, followed by cotton treated with an IST but not treated for TPB (Fig. 3, Supp. Table S3). Significantly more nymphs were observed in cotton without an IST compared to cotton treated with an IST when no insecticide applications were made for TPB. However, when insecticides were used for TPB, there was no difference in the number of nymphs observed regardless of using an IST (Fig. 3, Supp. Table S3). There was an interaction of trait and spray regimen on TPB nymphs (F=3.06; df=2, 846.7; P=0.047). In plots unsprayed or sprayed

automatically for TPB, significantly more nymphs were found in non-Bt cotton than the Bt Cry51Aa2 cotton. No difference in the number of nymphs was observed between Bt Cry51Aa2 cotton and non-Bt cotton when managed for TPB using the threshold approach (Fig. 3, Supp. Table S3).

For the average number of large TPB nymphs found per 3.05 row m during bloom, no interactions of IST by trait were found (F=3.14; df=1, 831.8; P=0.077) or IST by spray regimen (F=0.19; df=2, 831.7; P=0.824). However an interaction of trait by spray regimen was observed (F=21.23; df=2, 830.5; P<0.001). There were more large nymphs in non-Bt cotton not sprayed for TPB (5.31±0.33) followed by Bt Cry51Aa2 cotton not sprayed for TPB (2.64±.23). However, there was no difference in the number of large nymphs observed in non-Bt cotton sprayed on threshold or automatically (Fig. 3, Supp. Table S3). There were fewer large TPB nymphs found in the Bt Cry51Aa2/threshold treatment than the non-Bt/threshold treatment (Fig. 3, Supp. Table S3). For all spray regimens, significantly fewer nymphs were observed on Bt Cry51Aa2 cotton than on non-Bt cotton.

There were no significant main or interaction effects of IST, trait, or spray regimen for stink bugs or clouded plant bugs (Supp. Table S4). In untreated plots, an average of 0.13±0.02 stink bugs and 0.23±0.01 clouded plant bugs were found per 10 row feet during bloom.

Yield

For seed-cotton yield interactions of IST by trait (F=6.49; df=1,177; P=0.012) and trait by spray regimen were found (F=4.36; df=2, 177; P=0.014). Yields were higher when there was some type of thrips control, either an IST or the Bt trait, compared with non-Bt cotton without an IST (Fig. 4, Supp. Table S5). Cotton treated aggressively for TPB out-yielded the other spray regimens and there was no difference between the Bt Cry51Aa2 (4190±157.07 kg/ha) and non-

Bt cotton (4006±194.78 kg/ha). However, the Bt Cry51Aa2 cotton yielded approximately 17% more than the non-Bt cotton when it was not sprayed for TPB (Fig. 4, Supp. Table S6). There was not a significant difference in yield between the non-Bt cotton managed with automatic insecticide applications and the Bt Cry51Aa2 cotton managed using the threshold approach, and there was also no difference between the yields of Bt or non-Bt cotton managed using the threshold approach for TPB management (Fig. 4, Supp. Table S6).

Percent gin turnout averaged 42.3% and was not affected by any of the model parameters (Table 1). Trait had an effect on fiber length and uniformity (Table 1), with the average length of non-Bt cotton (3.05±0.01 cm) being longer than Bt Cry51Aa cotton (3.02±0.01 cm), and non-Bt (84.37±0.11%) cotton had higher uniformity than Bt Cry51Aa2 cotton (84.01±0.13%). The IST did not affect any fiber properties. For fiber length, when no seed treatment was used, fiber length was 3.05 cm compared to when an IST was used, fibers were 3.03 cm long. Non-Bt cotton generally had higher fiber strength than the Bt Cry51Aa2 cotton, but an interaction of trait and spray regimen on fiber strength was found where non-Bt cotton that was not sprayed for TPB was stronger (=34.35±0.31) than all other treatments (Table 1). The non-Bt/automatic spray regimen (=33.48±0.37) was not different than non-Bt/threshold (=33.11±0.25) or Bt Cry51Aa2/threshold (=32.89±0.24), but the non-Bt/automatic spray was stronger than the Bt Cry51Aa2/not sprayed (=32.62±0.32) and the Bt Cry51Aa2/automatic (=32.55±0.34) treatments (Table 1).

Micronaire was significantly affected by both trait and spray regimen (Table 1, Fig. 5, Supp. Table 7) Micronaire was higher in non-Bt cotton (4.64±0.04) than in Bt Cry51Aa2 cotton (4.54±0.04). Applying insecticides decreased micronaire. Cotton not treated for TPB had higher micronaire (4.76±0.05) than all other treatments. Cotton sprayed aggressively for TPB had

higher micronaire (4.58±0.04) than cotton sprayed based on threshold (4.43±0.04). Similarly, reflectiveness was also affected by trait and spray regimen (Table 9). A higher reflectance was found in Bt cotton (72.68±0.17) than in the non-Bt cotton (71.78±0.21), and cotton not sprayed for TPB had lower reflectance than treatments sprayed with insecticides. Trait did not affect yellowness, but a significant effect of spray regimen and a trait by spray regimen interaction was observed (Table 9). Generally, cotton not sprayed with insecticides was more yellow than cotton treated according to threshold or automatically, but this difference was only significant for the Non-Bt cotton (Fig. 5, Supp. Table S7).

Discussion

Based on injury ratings, vigor ratings, and biomass, Bt Cry51Aa2 provided as good or better protection against thrips than non-Bt cotton with an IST plus a foliar application of insecticide made at the first true leaf. When compared to plots without thrips control, Bt Cry51Aa2 cotton with no IST reduced total thrips numbers by 71.3%, while the non-Bt cotton with an IST and a foliar insecticide application reduced thrips numbers by 74.2%. Although the reduction in thrips populations was similar between these two treatment combinations, thrips injury ratings between these treatment combinations were significantly different. In terms of both thrips numbers and thrips injury ratings, there was a benefit of using an IST plus the foliar insecticide application in conjunction with the Bt Cry51Aa2. Imidacloprid, a main thrips-control component in the seed treatment used within these experiments (Aeris), is reported to have non-preference qualities for tobacco thrips (Joost and Riley 2005). Our field and laboratory trials have shown Bt Cry51Aa2 is non-preferred (unpublished data). Because at least some of the effect of Bt Cry51Aa2 is due to a non-preference, the effect on thrips might be exaggerated in small plot research where non-Bt cotton is in close proximity. However, the non-preference

aspect of imidacloprid is believed to have played a role in the delayed resistance of tobacco thrips to imidacloprid compared to thiamethoxam (Huseth et al. 2017). Having two non-preference modes of action (Bt Cry51Aa2 and imidacloprid) against tobacco thrips might help to delay possible resistance to this technology.

In both above ground biomass weight and vigor ratings, non-Bt cotton treated with an IST had more biomass and was more vigorous than the Bt Cry51Aa2 cotton without an IST. When comparing these data to thrips numbers and thrips injury, it suggests that something other than thrips caused this difference. Another component of Aeris is thiodicarb, which has activity on root-knot and reniform nematodes (Hall et al. 2017), and it provides some additional thrips control (Cook et al. 2017). Although it is possible that some benefit was provided from thiodicarb on plant vigor and above ground biomass, it is unlikely that the increase was associated with control of nematodes, as nematode are present at low levels in the fields where these trials were conducted. Although there appeared to be an added benefit of adding an IST and/or the foliar application of acephate to Bt Cry51Aa2, the increased biomass and vigor did not affect yield. Regardless, it was important to have early-season protection from thrips. Bt Cry51Aa2 cotton had more squares at the first week of bloom than non-Bt cotton, possibly reflecting earliness for the Bt Cry51Aa2 cotton due to thrips protection, which can play a role in TPB management later in the season. On average, cotton with only a base fungicide treatment yielded 11.5% less seed-cotton than treatments with some form of thrips control, either Bt Cry51Aa2 or a traditional insecticide approach. In our tests, there was a 181 kg per ha increase of yield when thrips infestations were managed, and this increase is consistent with a meta-analysis by North et al. (2016) who reported a 127 kg per ha increase in yield when a neonicotinoid seed treatment was used in cotton. This yield increase demonstrates the potential

importance of Bt Cry51Aa2 for thrips management, particularly when considering the documented occurrence of tobacco thrips resistance to neonicotinoid insecticides (Darnell 2015, 2016, Huseth et al. 2016).

Overall, pre-bloom TPB infestations were low to moderate. Averaged across all trials, an average of 3 bugs per 25 sweeps was observed in plots not treated for TPB. Fewer adults were found in the Bt Cry51Aa2 cotton than non-Bt cotton. Non-Bt cotton required over twice as many total insecticide applications (7) to manage these infestations than Bt Cry51Aa2 cotton (3). Pre-bloom square retention was adequate (> 80%) regardless of treatment, but in plots not sprayed with insecticides for TPB, square retention was higher in Bt Cry51Aa2 cotton than in non-Bt cotton. In fact, untreated Bt Cry51Aa2 plots had as good or better square retention than non-Bt plots that were managed with the threshold approach. It would be expected to have better retention in sprayed plots than unsprayed, but this was not the case. The difference in square retention suggests that other factors may be contributing to higher square retention in the Bt Cry51Aa2 cotton, perhaps non-preference, as observed with thrips. A non-preference affect may have implications on the efficacy of this technology on TPB when implemented in larger fields or for resistance management.

The interaction of IST by spray regimen on TPB nymphs found in cotton during bloom is not easily explained. Generally, more bugs were found on cotton treated with an IST. This could be explained by this cotton being more attractive because it had significantly better early season vigor than cotton treated with only a base fungicide seed treatment. However, this seems less likely considering there were no significant differences in total square counts during the first week of squaring for cotton with or without an IST during the first week of squaring, nor was there a differences in overall first position fruit retention at first bloom. No significant difference

in the average number of TPB nymphs was found between the Bt Cry51Aa2 and non-Bt cotton when they were managed according to threshold. However, across both locations and years, there were fewer total insecticide applications made during this window (11 vs. 8). There were significantly more large nymphs in the non-Bt cotton than the Bt Cry51Aa2 cotton. Large nymphs are especially important because larger nymphs feed more and subsequently cause more injury than small nymphs (Cooper and Spurgeon 2013). Seeing limited effects of Bt Cry51Aa2 on numbers of small nymphs would be expected if Bt Cry51Aa2 slowly killed nymphs and delayed their development. This is consistent with Baum et al. (2012), who reported smaller plant bug nymphs to be more sensitive to the Bt Cry51Aa2 protein than larger nymphs and adults and that mortality at field relevant rates required 6 days. It is also important to note the Bt Cry51Aa2 protein did not cause a high level of mortality and that some TPB nymphs survived to larger nymphs.

When not treated with insecticides for TPB, the Bt Cry51Aa2 cotton yielded more than non-Bt cotton, but there was a substantial yield increase when insecticides were applied to the Bt Cry51Aa2 cotton. Yields were similar between Bt Cry51Aa2 and non-Bt plots managed for TPB using the threshold approach but an average of 1.25 fewer insecticide applications (range 0-3) were made to the Bt Cry51Aa2 cotton (Figure 3). The use of this trait, especially in areas with high TPB pressure, may reduce the total number of insecticide application made during the growing season. However, proper scouting and timely applications of insecticides are still needed to manage TPB.

Differences in lint quality parameters appeared to be at least partially confounded by inherent differences between the Bt Cry51Aa2 and non-Bt lines, despite the varieties being near-isogenic. While use of an IST did not impact fiber quality parameters, spray regimen impacted

micronaire, fiber strength, yellowness, and reflectance. Micronaire and strength were higher in plots that were not sprayed for TPB. Higher micronaire and strength likely resulted from a higher percentage of harvestable bolls in these plots coming from early, more mature bolls, as plant bug infestations most likely affected mid- and late-season fruit. Differences in yellowness and reflectance were likely the result of differences in boll injury between the Bt Cry51Aa2 and non-Bt cotton and also between different insecticide regimens. These data indicate TPB injury reduced reflectance and increased yellowness. Although it is possible that stink bugs or clouded plant bugs could also affect fiber quality (Palakkaty-Thodi, et al. 2014), no significant difference in the numbers of these pests was observed among treatments, and generally low populations were found. Therefore, these differences were likely caused by TPB.

The evaluated Bt Cry51Aa2 trait provided partial control of thrips and TPB and thereby could reduce reliance on traditional insecticides to control infestations of these insects. This reduction would help preserve susceptibility to foliar applied insecticides and alleviate problems with secondary pest outbreaks that are induced by use of broad-spectrum insecticides which disrupt beneficial arthropod populations. Therefore, the Bt Cry51Aa2 trait could be an important component of an integrated pest management plan for both thrips and TPB.

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Appendix I

Table 1. Analysis of variance for the impact of treatment effects on fiber quality parameters when averaged across two years and two locations. IST = insecticide seed treatment, Trait = Bt Cry51Aa2 or non-Bt, and Spray = foliar insecticide regimen used to control tarnished plant bug.

Treatment Effect	*F*	df	*P*	*F*	df	*P*
	Gin Turnout			**Fiber Length**		
IST	2.79	1, 77	0.090	3.70	1, 77	0.058
Trait	0.69	1, 77	0.395	10.74	1, 77	0.002
IST*Trait	0.68	1, 77	0.414	0.33	1, 77	0.569
Spray	0.33	2, 77	0.722	1.09	2, 77	0.341
IST*Spray	1.56	2, 77	0.217	2.63	2, 77	0.078
Trait*Spray	2.12	2, 77	0.127	1.08	2, 77	0.345
IST*Trait*Spray	1.78	2, 77	0.175	0.00	2, 77	0.997
	Fiber Strength			**Fiber Uniformity**		
IST	0.02	1, 77	0.655	0.02	1, 84	0.896
Trait	29.56	1, 77	<0.001	4.39	1, 84	0.041
IST*Trait	0.54	1, 77	0.466	0.75	1, 84	0.388
Spray	3.25	2, 77	0.044	1.07	2, 84	0.352
IST*Spray	0.52	2, 77	0.599	0.12	2, 84	0.886
Trait*Spray	6.15	2, 77	0.003	2.11	2, 84	0.128
IST*Trait* Spray	0.21	2, 77	0.814	0.16	2, 84	0.849
	Micronaire			**Reflectiveness**		
IST	0.37	1, 77	0.546	3.39	1, 83	0.069
Trait	8.53	1, 77	0.005	19.29	1, 83	<0.001
IST*Trait	0.00	1, 77	1.00	0.86	1, 83	0.356
Spray	30.70	2, 77	<0.001	12.35	2, 83	<0.001
IST*Spray	1.73	2, 77	0.185	0.32	2, 83	0.730
Trait*Spray	0.70	2, 77	0.502	0.65	2, 83	0.527
IST*Trait*Spray	0.83	2, 77	0.441	1.85	2, 83	0.163
	Yellowness					
IST	0.01	1, 77	0.912			
Trait	0.24	1, 77	0.623			
IST*Trait	0.28	1, 77	0.595			
Spray	4.68	2, 77	0.012			
IST*Spray	0.38	2, 77	0.685			
Trait*Spray	3.22	2, 77	0.045			
IST*Trait*Spray	0.60	2, 77	0.551			

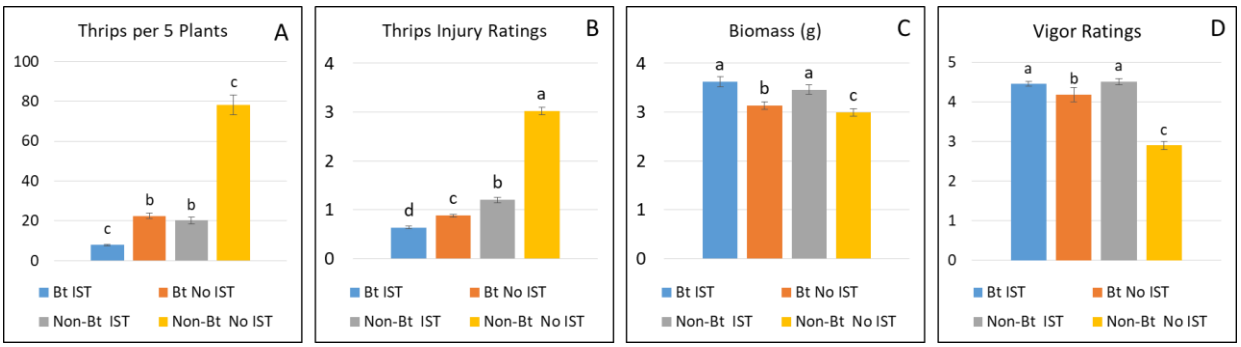

Figure 1. Average total number of (A) thrips per five plants, (B) average thrips injury rating, (C) average above ground biomass per five plants, and (D) average vigor ratings for Bt Cry51Aa2.34_16 and non-Bt cotton, with and without an insecticide seed treatment (IST) averaged across two years and two locations. Error bars represent 95% confidence intervals. Common letters above bars indicate treatments are not different (Fisher's Protected LSD, α=0.05). Visual rating of thrips injury on a 0 – 5 scale where 0 represents no injury to any plant in a plot. Visual rating of plant vigor on a 0 – 5 scale where 0 indicates no living plants in a plot.

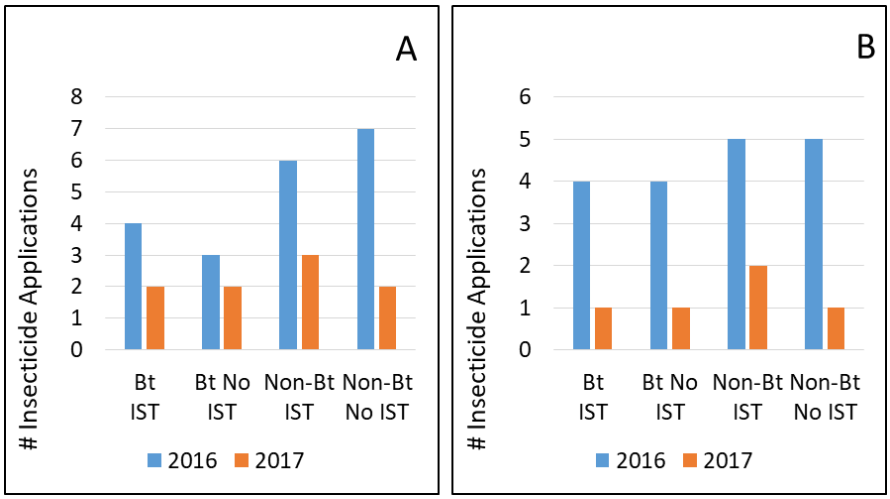

Figure 2. Total number of foliar insecticide applications made to manage tarnished plant bugs in Bt Cry51Aa2.34_16 and non-Bt cotton managed using a threshold approach, with and without an insecticide seed treatment (IST) in (A) Jackson and (B) Milan, TN.

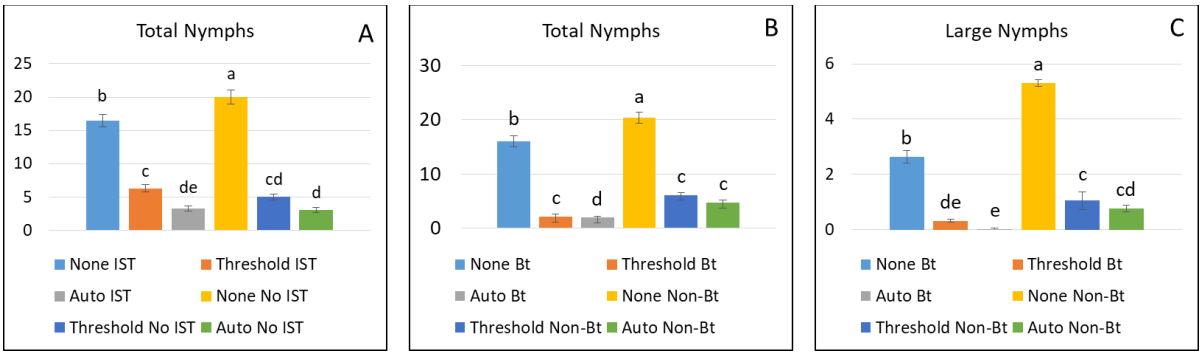

Figure 3. Average total number of tarnished plant bug nymphs found per 3.02 m row (SEM) of blooming cotton averaged across two years and two locations. (A) Cotton with and without an insecticide seed treatment (IST) and managed for tarnished plant bug with different spray regimens [automatic (auto), threshold (thresh) or none]. (B) Bt Cry51Aa2.834_16 and non-Bt cotton managed for tarnished plant bug with different spray regimens. Error bars represent 95% confidence intervals. Common letters above bars indicate treatments are not different (Fisher's Protected LSD, α=0.05).

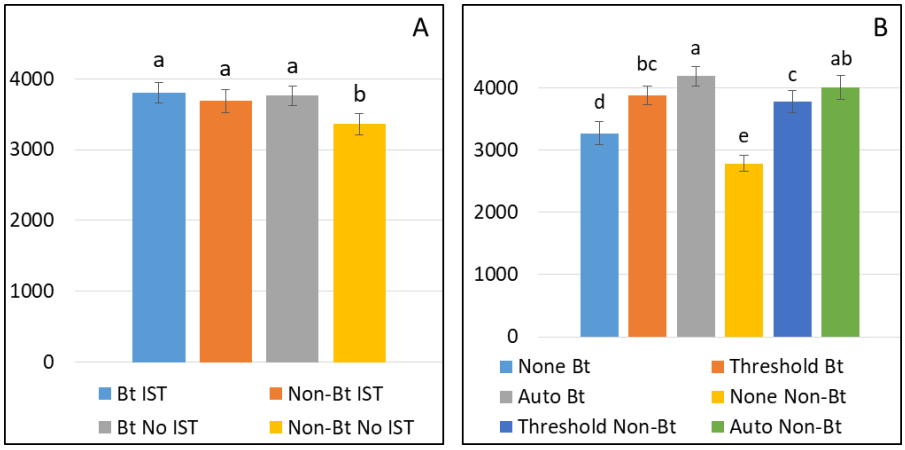

Figure 4. (A) Average total kilograms of seed-cotton per hectare for Bt Cry51Aa2.34_16 and non-Bt cotton treat with and without an insecticide seed treatment (IST) averaged across two years and two locations. (B) Average total kilograms of seed-cotton per hectare for Bt Cry51Aa2.34_16 and non-Bt cotton managed for tarnished plant bug with different spray regimens [automatic (auto), threshold (thresh) or none] averaged across two years and two locations. Error bars represent 95% confidence intervals. Common letters above bars indicate treatments are not different (Fisher's Protected LSD, α=0.05).

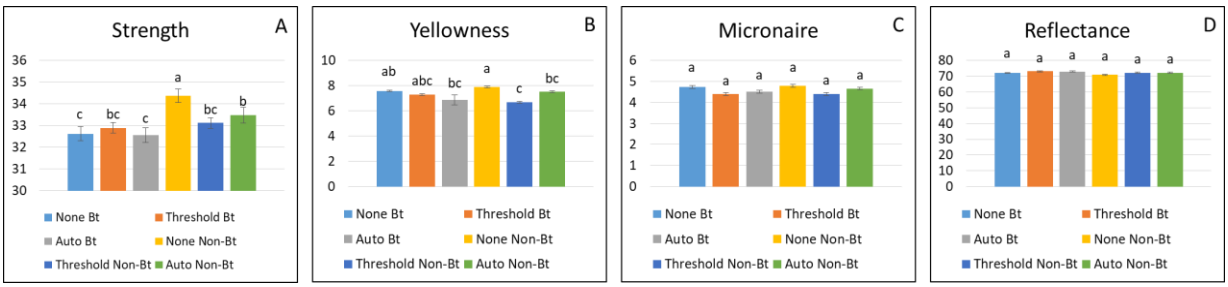

Figure 5. Influence of Bt Cry51Aa2.34_16 and non-Bt cotton managed for tarnished using different spray regimens [automatic (auto), threshold (thresh) or none] on average cotton fiber quality indices (SEM) averaged across two years and two locations. Error bars represent 95% confidence intervals. Common letters above bars indicate treatments are not different (Fisher's Protected LSD, α=0.05).

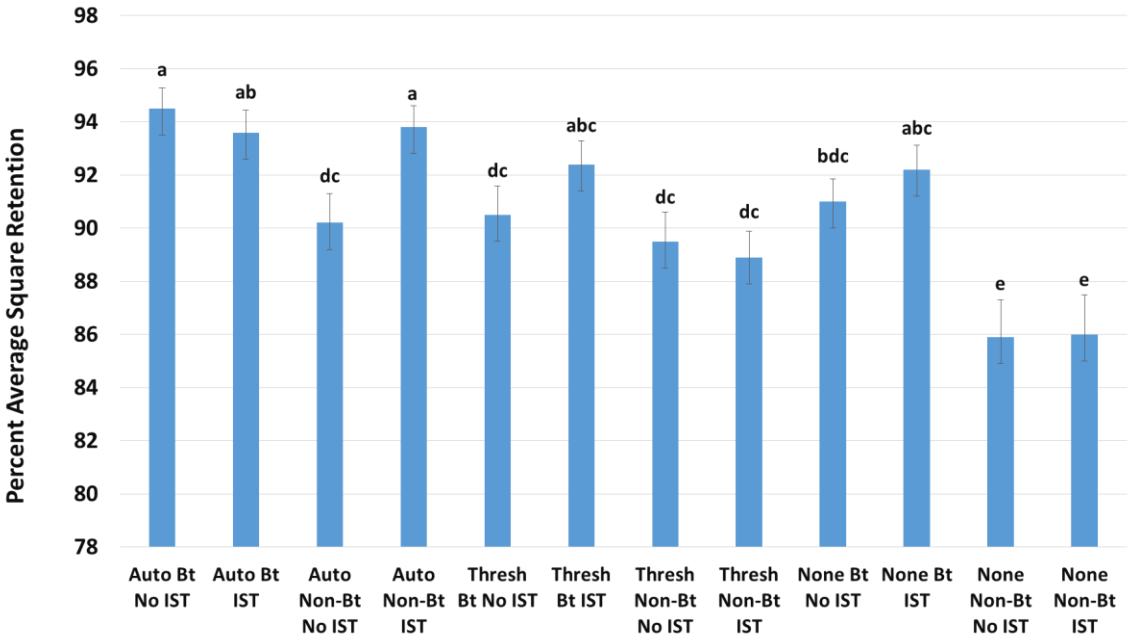

Figure 6. Average pre-bloom percent square retention graphed by 1) TPB spray regimens [automatic (auto), threshold (thresh) or none], 2) isoline [Cry51Aa2.34_16 (Bt) or non-Bt] and 3) insecticide seed treatment [insecticide seed treatment (IST) or no IST], averaged across two years and two locations. Error bars represent 95% confidence intervals. Common letters above bars indicate treatments are not different (Fisher's Protected LSD, α=0.05).

CHAPTER II

Behavioral Response of Thrips (Thysanoptera: Thripidae) and Tarnished Plant Bug (Hemiptera: Miridae) to a New Bt Toxin, Cry51Aa2.834_16, in Cotton

Abstract

Thrips (Thysanoptera: Thripidae) and tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae), are among the most important insect pests of cotton, *Gosssypium hirsutum*, in the mid-southern United States. These pests are currently managed primarily by insecticides, however a new Bt toxin, Cry51Aa2.834_16 is under evaluation for control of thrips and tarnished plant bug. Experiments were conducted in 2016 and 2017 to evaluate the behavioral response of thrips and tarnished plant bug to Bt Cry51Aa2.834_16. Adult thrips avoided Bt Cry51Aa2.834_16 cotton in field choice tests and field tests of cotton not treated with insecticides. In a greenhouse choice test more adult thrips and eggs were found on non-Bt cotton by approximately a 2:1 margin over Bt Cry51Aa2.834_16 cotton. Similarly, in a field test of non-treated Bt Cry51Aa2.834_16 and non-Bt cotton, 68% of adult thrips collected were found on non-Bt cotton. In cotton that was not sprayed with insecticides, Bt Cry51Aa2.834_16 did not affect the distribution of tarnished plant bug within the canopy, although more square and flower injury was caused by tarnished plant bug in non-Bt cotton. Adult tarnished plant bug exhibited a non-preference for diet containing lyophilized Bt Cry51Aa2.834_16 leaves and for excised Bt Cry51Aa2.834_16 squares in choice tests with non-Bt squares. The behavioral response of these pests when exposed to this new Bt toxin will play a key role in the efficacy and potential resistance management strategies if this new technology is incorporated in an overall cotton insect pest management system.

Keywords: Thrips, Tarnished Plant Bug, Bt Cotton, Cry51Aa2.834_16, Behavior

Introduction

Thrips (Thysanoptera: Thripidae) are the most important insect pests of seedling cotton in the Mid-Southern United States (Cook 2018). The tobacco thrips, *Frankliniella fusca* (Hinds), is the dominant species found on cotton in the Mid-South (Reed and Jackson 2002, Layton and Reed 2002, Cook et al. 2003, Reed et al. 2006), often composing more than 90% of all thrips

collected on seedling cotton (Reed et al. 2006, Stewart et al. 2013). When left untreated, thrips injury can lead to stunted growth, delayed maturity, reduced stands, and yield loss (Layton and Reed 2002, Stewart and Lentz 2010). Thrips are managed using neonicotinoid seed treatments or prophylactic in-furrow insecticide applications. Imidacloprid and thiamethoxam are the primary seed treatments used by cotton growers (Cook et al. 2011). In recent years, failures of thrips control with neonicotinoid seed treatments have occurred across the Mid-South (Darnell et al. 2018) and the Southeast (Huseth et al. 2016, Huseth et al. 2017), leading to the need of new ways to manage this pest. The tarnished plant bug, *Lygus lineolaris* (Palisot De Beauvois) (Hemiptera: Miridae), is the key insect pest of cotton in the Mid-South. Economic damage from tarnished plant bug occurs from the beginning of squaring (flower buds) and continues through bloom (Layton 2000). Injury results from the abscission of squares and bolls, often leading to substantial yield loss (Scales and Furr 1968, Scott et al. 1985, Layton 1995, Russell 1999). Tarnished plant bug infestations are managed using foliar insecticide applications, however the tarnished plant bug has developed resistance to many of the insecticides commonly used for management (Cleveland and Furr 1979, Snodgrass and Scott 1988, Snodgrass 1996, Snodgrass and Scott 2000, Snodgrass 2006, Snodgrass et al. 2009, Parys et al. 2017). Resistance issues have made tarnished plant bug infestations in cotton difficult to manage and new ways to combat this pest are required to improve the profitability of the crop.

Cotton varieties expressing *Bacillus thuringiensis* (Bt) have been widely adopted for controlling key lepidopteran pests (Siebert et al. 2008). Although no commercial cotton varieties expressing Bt toxins for control of hemipteran or thysanopteran pests are currently available, Monsanto Company (St. Louis, MO) has developed a new Bt protein, Cry51Aa2.834_16 (referred hereafter as Bt Cry51Aa2) with activity against thrips and tarnished plant bug (Baum et

al. 2012, Gowda et al. 2016, Bachman et al. 2017, Graham and Stewart 2018). Graham and Stewart (2018) reported that cotton expressing Cry51Aa2 showed as good or better thrips control than a current, insecticide based approach for thrips management. The authors also found that when sprayed based on tarnished plant bug thresholds, Bt Cry51Aa2 cotton and non-Bt cotton made similar yields, but the Bt required fewer insecticide applications. While these studies suggest that this technology could be an effective management strategy, no studies have been published on the behavioral effects of Cry51Aa2 on thrips or tarnished plant bugs in cotton.

Tobacco thrips have been reported to have an avoidance behavior based on insecticide use or plant types. When given a choice, they prefer leaves not treated with imidacloprid and probe and ingest less frequently while feeding (Joost 2003). When given a choice, tobacco thrips have shown a preference of chickweed, *Stellaria media* (L.), over tomato, *Solanum lycopersicum* (L.), for oviposition, but interestingly, tobacco thrips laid significantly more eggs in tomato plants than chickweed in a no choice test (Chaisuekul and Riley 2005).

The distribution of tarnished plant bugs within cotton plants is an important factor for both sampling techniques and management of this pest. On average, 75% of both nymphs and tarnished plant bug adults are found on the mainstem terminal and fruit and vegetative structures of the upper six nodes of the cotton plant (Snodgrass 1998). Prior to bloom, nymphs are found on fruiting structures, while adults tend to be on vegetative structures. As the crop matures to bloom, adults tend to become more dispersed throughout the reproductive and vegetative structures of the plant (Snodgrass 1998). Studies conducted to determine behavioral responses of tarnished plant bug to insecticide applications within the cotton canopy have shown mixed results. Graham (2016) showed no effect of insecticide treatment on the distribution of tarnished plant bug. Fontenot (2009), however, found that significantly more tarnished plant bugs were

found in the middle third of the canopy in acephate-treated cotton plants compared to untreated cotton plants. The effects on tarnished plant bug distribution within the plant canopy from transgenic cotton plants expressing Bt Cry51Aa2 have not been reported. It is known that the distribution of some insects can be affected by the expression of Bt cry toxins. The cotton bollworm, *Helicoverpa zea* (Boddie), has been shown to avoid structures that express high levels of Bt proteins (terminals and squares) and tend to feed more on structures with lower expression (flowers and bolls) (Greenplate 1999, Adamczyk et al. 2001, Akin et al. 2002, Gore et al. 2002). Gore et al. (2002) showed that bollworm larvae were more likely to move from the plant structure they were placed on in Bt cotton plants than non-Bt plants. While expression levels of Bt Cry51Aa2 have not been reported for individual structures of the cotton plant, it is known that the protein is present throughout the cotton plant (Baum et al. 2012).

The behavioral responses of thrips and tarnished plant bug to Bt Cry51Aa2 may play an important role in how cotton is managed for these important pests. Avoidance of Bt Cry51Aa2 expressing plants or of higher expressing parts of a plant could affect the efficacy of this toxin on thrips or tarnished plant bug. Previous field studies by Graham and Stewart (2018) showed that this toxin reduced thrips densities on seedling plants. Further, plant protection that is at least partly based upon avoidance has potential impact on resistance management strategies. The objective of this study was to evaluate the effects of cotton plants expressing Bt Cry51Aa2 on thrips and tarnished plant bug behavior.

Materials and Methods

Thrips

Choice Test (Field). Choice tests were done in 2016 and 2017 at the West Tennessee Research and Education Center (WTREC) in Jackson, TN to determine if field populations of

adult thrips showed a preference for non-Bt cotton compared to cotton expressing Bt Cry51Aa, in part because previous field studies showed that this toxin reduced thrips densities on seedling plants (Graham and Stewart 2018). In both years, greenhouse trays with 36 cells in each tray were planted with Bt Cry51Aa2 and non-Bt near-isogenic lines of Deltapine 393 (Monsanto Company, St. Louis, MO). Each tray had a total of 40 seeds planted, 20 Bt Cry51Aa2 and 20 non-Bt with Bt Cry51Aa2 seeds on one side and non-Bt on the other. Seeds were planted in potting soil roughly 2.0 cm below the soil surface and watered. In 2016, seeds were planted on 20 May an on 15 May in 2017. Trays were placed in an incubator set at 29°C and 40-60% RH with a 14 h light and watered as needed. When seedlings reached the first true leaf stage, eight trays containing cotton seedlings were placed on soil in a fallow field of the experiment station for 24 h to allow natural infestation and oviposition by thrips. In each year trays were considered replications with eight replications per year. After 24 h, 10 Bt Cry51Aa2 and non-Bt seedlings were collected from each tray by cutting the seedlings at the soil surface and placing in jars containing a 70% ethyl alcohol and water mixture to collect adult thrips from them. Each plant was removed from the jar and rinsed with 70% ethyl alcohol over a glass container topped with a sieve (150 μm) to collect adult thrips. Jars were then rinsed with 70% ethyl alcohol over the sieve to collect any remaining thrips left inside. The sieve was then rinsed with 70% ethyl alcohol into a gridded 100 mm x 15 mm petri dish and adult thrips were counted underneath a microscope using 10-20x magnification, and sight identified as either tobacco thrips, soybean thrips *Neohydatothrips variabilis* (Beach) or other thrips (i.e. not tobacco thrips or soybean thrips).

The remaining 10 Bt Cry51Aa2 and non-Bt seedlings in each tray were placed in an insect rearing room. Seedlings were cut at the soil level, and three plants of the same isoline were

placed in 50 ml centrifuge tubes suspended over a bowl of water and modified to allow plant stems to reach water to prevent desiccation. The bottoms of the tubes were covered with plumber's putty and the tops were capped to keep newly-emerged thrips in tubes and to keep water from seeping into the tubes. Samples were left in the insect rearing room at a temperature of ~27°C, at 40-60% relative humidity, and 14 h of light per day for 5 d, allowing eggs to hatch and immature thrips to emerge. Seedlings were removed from tubes and both tubes and seedlings were rinsed with 70% ethyl alcohol over a glass container topped with a sieve to collect immature thrips. Finally, the sieve was rinsed with ethyl alcohol into a gridded petri dish, and the numbers of immature thrips were counted in order to evaluate any ovipositional preferences of adult thrips for Bt Cry51Aa or non-Bt cotton.

Choice Test (Greenhouse). To further evaluate the ovipositional response of tobacco thrips to Bt Cry51Aa2 in cotton, a greenhouse study using laboratory-reared tobacco thrips was conducted in 2017 at WTREC. The tobacco thrips colony was reared at Mississippi State University on pieces of cabbage in a rearing room at a temperature of ~27°C, at 40-60% relative humidity, and 14 h of light per day in 5.1 L Berry Thinwall Containers (PFS Sales Co., Raleigh, NC). Each pot was planted with 2 non-Bt seeds and 2 Bt Cry51Aa2 seeds for a total of 4 seeds per pot and six pots per treatment. Pots used were similar 5.1 L Berry Thinwall Containers with lids. The lids were modified to allow for ventilation but prevent thrips from escaping by cutting a circular hole, approximately 10 cm diameter, and covering the hole with 100 micron nylon mesh (Midwest Filter Corporation). Similar holes were cut in the bottom of the pots and covered with the same mesh to keep soil from being water logged. Seeds were planted in potting soil about 2.0 cm below the soil surface, and grown in a greenhouse. Upon full expansion of the first true leaf, pots were infested with 10 adult tobacco thrips from the laboratory colony described. Caged

thrips were kept in the greenhouse for 5 d to allow for oviposition. Six pots were used with each pot considered a replication. To determine the influence of Bt Cry51Aa2 on oviposition, leaves were cut from the stems and were decolorized by boiling 3-5 minutes following the lacto-phenol acid fuschin staining technique detailed by Nuessly et al. (1995) and Parella and Rob (1982). Stained leaves were cooled for 3-5 h and examined under a dissecting microscope as described by Chitturi (2005) after excess stain was removed with warm water. The total number of eggs was recorded for each treatment.

Field Test. Small-plot replicated field tests were established to evaluate the influence of Bt Cry51Aa2 on thrips. Cotton was planted in May 2016 and 2017 at WTREC and at the Research and Education Center at Milan, TN (MREC). Near-isogenic lines of DP393, either Bt Cry51Aa or non-Bt cotton, were planted and treatments were arranged as described by Graham and Stewart (2018). Thrips were sampled at the 1.5 and 3.5 true leaf stage from plots that were not treated with seed or foliar applied insecticides. Thrips were collected from seedlings and counted as previously described.

Tarnished Plant Bug

Field Behavior. An additional field experiment was conducted at WTREC in 2017 to determine the effects of cotton varieties expressing Bt Cry51Aa2 on the distribution behavior of tarnished plant bug. Plots were planted with near-isogenic lines of DP393, one expressing Bt Cry51Aa2 and one non-Bt isoline. The experiment was planted 9 May at a seeding rate of 13.2 seeds/m. The experiment was designed as a randomized complete block (RCB) with four replications. To prevent confounding effects of thrips damage, all cotton was treated with a commercial rate of imidacloprid and thiodicarb as an insecticide seed treatment, Aeris (Bayer CropScience, Raleigh, NC). Individual plots were 10.7 m long and 4 rows wide with 0.97 m row

spacing. Beginning at first-square and until first flower, plots were scouted and managed for tarnished plant bug based on the *Insect Control Recommendations for Field Crops* for Tennessee (PB 1690 Stewart and McClure 2017). No insecticide applications were made after flowering began.

During the second week of bloom, three sampling methods were used to collect data about tarnished plant bug density, locations within plants, and damage. The location of tarnished plant bugs was mapped on plants, by fruiting structure and node through visual examination until 25 bugs were found in each plot. Visual sampling of each individual plant began at the terminal and moved down each node and across to each fruiting structure on the respective nodes. Numbers of tarnished plant bug adults and nymphs were recorded by the node, position of fruiting structure, and type of fruiting structure (square, flower, or boll) as well as the total number of plants per plot required to find 25 bugs. Nymphs were classified based on size as either small (1^{st} and 2^{nd} instar), medium (3^{rd} and 4^{th} instar) or large (5^{th} instar). The relative density of tarnished plant bugs and various stink bug species (Hemiptera: Pentatomidae), two drop cloth samples were then taken in each plot by laying a black cloth (between two rows of cotton near the center of the plot) and vigorously shaking the plants from each row, in each sample the number of hemipterans per 3.02 m row were counted, and species were totaled separately..

Damage caused by tarnished plant bug infestations were assessed by visually sampling 25 random squares and 25 random flowers. A ‘dirty square’ showed signs of feeding from tarnished plant bug as a yellow staining and a ‘dirty flower’ shows signs of damaged anthers, petals and/or staining from tarnished plant bug excrement. Visual ratings of dirtiness were characterized based on subjective qualitative ratings as either low, medium, or high based on intensity of the injury.

The total number and life stages of tarnished plant bugs found in the squares and flowers were also recorded in each plot. A second rating was done two weeks after the initial rating and 25 flowers and thumb-sized bolls (≈ 2.3 - 2.8 cm diameter) were examined for tarnished plant bug injury. Bolls were assessed for puncture marks (stains) on the outside of bolls, stains on the inside of bolls, warts on the inside of bolls, and a visual estimate of percent damage of the developing lint. The severity of external boll staining was also rated on a 0 – 3 scale, with 0 being no injury and 3 being high injury. Boll injury caused by stink bugs could not be differentiated because injury symptoms are similar (Greene et al. 2006), but stink bugs densities were generally low in this experiment. Two drop cloth samples were taken in each plot to estimate tarnished plant bug and stink bug densities. The center two rows of each plot were harvested to determine the level of yield protection from Bt Cry51Aa2 when no insecticide applications are made for tarnished plant bug after the initiation of bloom.

Ovipositional Cage Test. A cage test was conducted at WTREC in 2017 to determine if tarnished plant bug oviposition was effected by Bt Cry51Aa2. Cotton seed, Bt Cry51Aa2 and non-Bt near-isogenic lines of DP393, was provided by Monsanto Co. (St. Louis, MO). Seeds were planted in a potting soil mix with one plant per pot. Plants were grown inside a greenhouse at WTREC until they were 8-9 nodes in size, having multiple squares but no flowers or bolls. Two plants, one Bt Cry51Aa2 and one non-Bt, were randomly placed in opposite corners in each of the eight cages. Cages were considered replications and were 61cm x 61cm x 121cm and covered with a 24 x 20 mesh polyester netting (BioQuip Products, Rancho Domingues, CA) and each cage was considered a replicate. A total of 15 tarnished plant bug adult females and 5 tarnished plant bug adult males were introduced into each cage. Insects were obtained from a laboratory colony reared at Mississippi State University and maintained on an artificial diet

developed by Cohen (2000). Adults were approximately 7-d old when placed in cages so that females were likely mated and reproductive. Insects were left in cages for 5 d, after which, plants were removed, taken to the laboratory, and examined for eggs. The upper five nodes of plants were examined under a dissecting microscope so that the number of eggs, which are typically embedded into the plant tissue (Fleischer and Gaylor 1988), could be counted. The location of eggs was categorized by nodal location and plant part (stem, leaf petioles, leaf, leaf veins, and squares).

Laboratory Tests. A laboratory test was also conducted in 2017 to determine if tarnished plant bugs exhibited an avoidance response when exposed to Bt Cry51Aa2. A total of 25 tarnished plant bugs were placed in 2.12-liter plastic rectangular containers with self-sealing lids (S.C. Johnson & Son, Inc., Racine, WI). Insects were from the same laboratory colony as described previously. The containers were modified by removing the lid, except for the sealing frame and replacing the removed portion with a tulle fine mesh screen. Containers were considered replications. Lyophilized leaf tissue of near-isogenic DP393 Bt Cry51Aa2 and non-Bt cotton were incorporated into the artificial diet developed by Cohen (2000). We tried to emulate a field rate of Cry51Aa2 toxin in the artificial diet present in leaf tissue (5.11g leaf tissue/ 350 ml diet) based on the LC_{50} for nymphs of *Lygus hesperus* (Knight) as reported by Bachman et al. (2017). Diet packs containing Bt Cry51Aa2 and non-Bt lyophilized leaves were placed on opposite sides of the containers on top of the mesh screen. Thus, tarnished plant bugs in each container had the option of feeding on one Bt Cry51Aa2 or one non-Bt diet pack filled with equal parts of lyophilized plant tissue. The orientation of the containers was randomized within a rearing room maintained at 27 ± 2°C, 60 ± 5% RH, and 14:10 L:D. Tests were done with first and third instars and 6-d old adults, with three or four replications (containers) for each test. The

number of adult tarnished plant bugs on diet packs were examined at 1, 3, 18, and 24 h intervals, first instars were examined at 1, 3, and 5 h intervals and third instars were examined at 1, 4, 8, and 24 h intervals. Diet packs given to adult tarnished plant bugs were taken at the end of the 24-h period and examined for the number of eggs laid into each pack.

Another laboratory study was conducted in 2018 using excised squares from Bt Cry51Aa2 and non-Bt cotton plants grown in a greenhouse at WTREC. Adult tarnished plant bugs were collected locally from wild hosts, mainly *Amaranthus palmeri* (S. Watson). Tarnished plant bugs were starved for 2 h prior to the start of the study. A total of four adult tarnished plant bugs were placed into 0.94-liter plastic containers with self-sealing lids. Each container had two Bt Cry51Aa2 and two non-Bt squares placed in the container corners. Containers were randomly arranged with a total of seven replications. Tarnished plant bugs were left in containers for 24 h, and the number of tarnished plant bugs on Bt and non-Bt squares was recorded at 1, 4, 8, and 24 h intervals. The number of eggs deposited in squares was also recorded after 24 h.

Statistical Analyses

Thrips. Adult thrips preference data, immature emergence data, and ovipositional preference data for choice tests were analyzed using a general linear mixed model of analysis of variance PROC GLIMMIX of SAS (Version 9.4, SAS Institute, Cary, NC). Bt trait (Bt Cry51Aa2) was a fixed effect in the model. Sample date, replication, and replication nested within sample date were random effects in the model to test whether significantly more numbers of adults were found on either variety, and to examine how many immatures resulted from eggs laid during the exposure period. Replication was included as the random effect for ovipositional preference data.

For the field test, a chi-square analysis was done to test if the distribution of adult thrips differed between the Bt Cry51Aa2 and non-Bt seedlings when not treated with an insecticide seed treatment by using PROC FREQ of SAS (Verison 9.4, SAS Institute, Cary, NC). Adult thrips numbers were analyzed using a general linear mixed model of analysis of variance PROC GLIMMIX of SAS (Version 9.4, SAS Institute, Cary, NC). Bt trait was considered a fixed effect. Year, location, year by location, and replication nested within year by location were designated as random effects to allow inferences to be made over a range of environments (Carmer et al. 1989, Blouin et al. 2011).

Tarnished Plant Bug. All field data were analyzed using a general linear mixed model of analysis of variance PROC GLIMMIX of SAS (Version 9.4, SAS Institute, Cary, NC). Bt trait was considered a fixed effect in all models. Bt trait, node, fruit position, and fruit structure were fixed effects and plant and plant nested within replication were included as random for tarnished plant bug plant distribution data. Sample date and replication were included as random for injured cotton squares, flowers and drop cloth data. The proportions of tarnished plant bugs on diet packs and squares were calculated at each rating interval. Laboratory bioassay data over the 24-h rating period were analyzed using a mixed model analysis of variance for repeated measures (PROC MIXED, Littell et al. 1996). Bt trait was considered a fixed effect and replication was considered random with time as the repeated measure.

In all statistical analyses, degrees of freedom were estimated using the Kenward-Rogers method (Kenward and Roger 2009). Means were estimated using LSMEANS and were separated using Fisher's protected least significant difference ($\alpha = 0.05$).

Results

Thrips

Choice Test (Field). The Bt trait reduced number of adult thrips found per ten plants (F = 28.00; df = 1, 29; $P < 0.001$) in the field choice test. On average, 8.44 ± 0.82 (mean ± SEM) thrips were found on non-Bt seedlings and 3.81 ± 0.67 were found on Bt Cry51Aa2 seedlings. Bt trait also reduced the number of tobacco thrips (F = 24.55; df = 1, 22; $P < 0.001$), as more tobacco thrips were found on non-Bt seedlings (6.13 ± 0.59) than on Bt Cry51Aa2 seedlings (2.31 ± 0.54). No difference was observed in the number of soybean thrips (F = 2.48; df = 1, 15; $P = 0.14$) or other thrips (F = 1.57; df = 1, 22; $P = 0.22$) (Table 3). The Bt trait did not affect the number of immature thrips that emerged from cotton naturally infested by adult thrips (F = 2.75; df = 1, 15; $P = 0.12$). On average, 10.88 ± 4.82 and 3.38 ± 1.65 immature thrips were found per 10 non-Bt plants and 10 Bt Cry51Aa2 plants, respectively.

Choice Test (Greenhouse). More eggs (F = 25.29; df = 1, 10; $P < 0.001$) were found in non-Bt (157.17 ± 12.24) than in Bt Cry51Aa2 cotton (72.67 ± 11.51) in the greenhouse choice test.

Field Test. The proportions of adult tobacco, soybean, and other thrips were nearly identical in Bt Cry51Aa2 and non-Bt cotton (X^2 = 2.34; df = 2; $P = 0.84$) (Table 3). However, the Bt trait affected the number of adult thrips per five plants (F = 29.53; df = 1, 187; $P < 0.001$), as more adult thrips were found on non-Bt cotton than on Bt Cry51Aa2 cotton. The Bt trait had a similar effect on all thrips species monitored (tobacco thrips F = 23.79; df = 1, 165; $P < 0.001$, soybean thrips F = 11.47; df = 1, 152.5; $P < 0.001$, and other thrips F = 12.51; df = 1, 152.6; $P < 0.001$) (Table 3).

Tarnished Plant Bug

Field Behavior. The Bt trait did not affect the distribution of tarnished plant bugs within the cotton canopy (F = 0.26; df = 1, 177.7; P = 0.61). Tarnished plant bugs were found on a mean of 6.2 nodes from the terminal on Bt Cry51Aa2 cotton and 6.0 nodes on non-Bt cotton. The Bt trait also did not affect average fruiting position where tarnished plant bugs were found (F = 1.23; df = 1, 177.4; P = 0.27) (Fig. 7). No interaction of Bt trait by fruiting structure was observed on the number of adult tarnished plant bugs found on fruiting structures (F = 2.03; df = 5, 185.1; P = 0.08). However, an interaction of Bt trait by fruit structure was observed for the number of nymphs found per structure (F = 5.06; df = 5, 184.6; P < 0.001). More nymphs were found in non-Bt flowers than were found on Bt Cry51Aa2 squares, Bt Cry51Aa2. No difference was observed between the number of nymphs found on non-Bt flowers, non-Bt bolls, non-Bt squares, and Bt Cry51Aa2 flowers (Fig. 8). Also, no difference was found in the numbers of tarnished plant bugs observed on non-Bt squares, Bt Cry51Aa2 flowers, Bt Cry51Aa2 squares, and Bt Cry51Aa2 bolls. Fewer nymphs were found on Bt Cry51Aa2 bolls than all other structures. Finally, Bt trait had no effect on the size of plant bug nymphs observed (Table 4). More plants (F = 4.11; df = 1, 189.1; P = 0.044) were required in Bt Cry51Aa2 plots (8.96 ± 0.91) than in non-Bt plots (7.59 ± 0.91) to locate the 25 tarnished plant bugs per plot.

More dirty squares (F = 21.24; df = 1, 6; P < 0.001) were found in non-Bt cotton (8.0 ± 0.71) than Bt Cry51Aa2 cotton (3.3 ± 0.75). However, no differences were observed in the mean number of adult (F = 0.20; df = 1, 6; P = 0.67) or immature tarnished plant bugs (F = 3.25; df = 1, 6; P = 0.12) found on squares (Table 5). Significantly more tarnished plant bug adults (F = 12.70; df = 1, 14; P = 0.003) and nymphs (F = 23.17; df = 1, 14; P < 0.001) were observed in non-Bt flowers than in Bt Cry51Aa2 flowers (Table 5). As a result, the Bt trait had an effect on the number of injured flowers (F = 30.31; df = 1, 14; P < 0.001), flowers with no injury (F =

31.59; df = 1, 14; $P < 0.001$), low injury ($F = 4.94$; df = 1, 14; $P = 0.04$), and high injury ratings ($F = 90.43$; df = 1, 14; $P < 0.001$). Flowers from Bt Cry51Aa2 cotton had less tarnished plant bug injury and substantially less severe injury than those from non-Bt cotton (Table 7).

More adults ($F = 5.09$; df = 1, 13; $P = 0.04$) were observed on drop cloth samples in non-Bt cotton (0.88 ± 0.39) than in Bt Cry51Aa2 cotton (0.13 ± 0.13) (Table 7). However, nymphs composed the vast majority (≈ 97%) of the overall tarnished plant bug population, and there was no effect of the Bt trait on the mean number of tarnished plant bug nymphs ($F = 0.69$; df = 1, 13; $P = 0.42$) or total tarnished plant bugs observed ($F = 0.36$; df = 1, 13; $P = 0.59$) (Table 6). The green stink bug, *Chinavia hilaris* (Say), and brown stink bug, *Euschistus servus* (Say), were the primary stink bug species observed, but the total numbers of stink bugs observed in non-Bt cotton and the Bt Cry51Aa2 cotton was similar (Table 7).

Similar to flower injury, the Bt trait reduced the level and severity of boll injury compared with non-Bt cotton. Thumb-sized bolls from Bt Cry51Aa2 plants had a lower number of stains on the outside of bolls and a lower severity of boll staining (Table 8). Similarly, more inner boll stains, more warts, and more lint staining was observed in non-Bt bolls compared with Bt Cry51Aa2 bolls (Table 8). Differences in square, flower, and boll injury were reflected by seed cotton yield (F = 35.38; df = 1, 3; P = 0.009), with higher yield in Bt cotton (2598 ± 175 kg/ha) compared with non-Bt cotton (1654 ± 126 kg/ha).

Ovipositional Caging Test. . In the adult tarnished plant bug cage test, no interaction of Bt trait by plant structure was found on the number of tarnished plant bug eggs laid per plant (F = 2.37; df = 4, 99.75; P = 0.06), nor was there an effect of the Bt trait (F = 3.12; df = 1, 99.75; P = 0.08). However, an effect of plant structure (F = 58.66; df = 1, 99.75; P < 0.001) was observed for the average number of eggs per plant, with the most tarnished plant bug eggs found in

petioles (29.9 ± 3.15) followed by leaves (8.7 ± 1.28). No differences were observed between the average number of tarnished plant bug eggs found in leaf veins (3.9 ± 0.63), squares (3.2 ± 0.62), or main stems (1.9 ± 0.62). On average, 54.1 ± 6.27 tarnished plant bug eggs were found in non-Bt cotton and 42.1 ± 6.18 tarnished plant bug eggs were found in Bt Cry51Aa2 cotton; however, this difference was not significant (F = 1.86; df = 1, 14; P = 0.19). In the study with excised squares, the Bt trait also did not affect the number of tarnished plant bug eggs laid (F = 2.64; df = 1, 6; P = 0.16), although there was a trend of more eggs in non-Bt squares (9.9 ± 5.05) than in Bt Cry51Aa2 squares (1.7 ± 0.91).

Laboratory Tests. Adult tarnished plant bugs preferred to feed on diet packs and excised squares that did not contain Bt tissue (Diet packs F = 28.34; df = 1, 84; P < 0.001 and Excised Squares F = 14.04; df = 1, 54; P < 0.001). There was no interaction of time by Bt trait on the proportion of adults observed on Bt diet packs or excised cotton squares (P > 0.05). More adults were observed on non-Bt diet packs than on Bt Cry51Aa2 diet packs, and on non-Bt squares than on Bt Cry51Aa2 squares (Fig. 9). Bt trait had no effect on the feeding choice of first instar (F = 0.94; df = 1, 16; P = 0.35), third instar (F = 0.53; df = 1, 230; P = 0.47), or nymphs overall (F = 0.53; df = 1, 48; P = 0.28) (Fig. 9). Similar to the difference in adult preferences, more tarnished plant bug eggs were found in non-Bt diet packs (44.0 ± 11.24) than Bt Cry51Aa2 diet packs (26.3 ± 8.99) (F = 30.87; df = 1, 2; P = 0.03).

Discussion

Thrips preferred non-Bt cotton over cotton expressing Bt Cry51Aa2, as evidenced by our choice studies done in the field and greenhouse where there was approximately a 2:1 preference for non-Bt cotton by adults and for oviposition. No previous research has reported the behavioral response of tobacco thrips to Bt Cry51Aa2; however, it is known that thrips have behavioral

responses to insecticides and plant types (Joost 2003, Chaisuekul and Riley 2005); tobacco thrips have been shown to avoid imidacloprid-treated leaves (Joost 2003) and to have an ovipositional preference to for tomato over chickweed (Chaisuekul and Riley 2005). We speculate that this avoidance behavior at least partly explains why imidacloprid seed treatments in cotton still provide better protection against thrips than thiamethoxam (Cook et al. 2016), despite assays indicating tobacco thrips are resistant to both insecticides (Darnell et al. 2018, Huseth et al. 2016). Thrips avoidance of cotton expressing Bt Cry51Aa2 appears to be a major mechanism of plant protection that has previously been observed in field trials (Graham and Stewart 2018). If most of Bt Cry51Aa2 activity on thrips is related to avoidance, it may have implications on insecticide resistance management, although the specific impacts are not clear.

Based on our observations, the presence of Bt Cry51Aa2 did not impact the distribution of tarnished plant bug within the canopy of cotton, although we had to scout ≈ 15% more plants to find similar numbers of bugs. This finding differs from the response of bollworm to lepidopteran-active Bt toxins in cotton, which move to avoid structures with high expression of toxins (Greenplate 1999, Adamczyk et al. 2001, Akin et al. 2002, Gore et al. 2002). In our study, nearly 66% of all tarnished plant bugs observed were on squares, regardless of whether they were on a Bt or non-Bt plant. This finding is similar to the results of Pack and Tugwell 1976, Snodgrass 1998 and Fontenot 2009. The majority of tarnished plant bugs were found on the upper six nodes of the cotton canopy, which is also consistent with studies by Snodgrass et al. (1998) and Graham (2016). Understanding the effect of Bt Cry51Aa2 on tarnished plant bug distribution is important because distribution plays a role in scouting techniques and insecticide efficacy. Because we found no effect of Bt Cry51Aa2 on the distribution of tarnished plant bug dispersal on the cotton plant, current sampling strategies should still be effective. Sumner and

Herzog (2000) showed that foliar insecticide applications provided less control of tarnished plant bugs in the lower and middle parts of the canopy. Our data suggest that insecticide applications should be equally effective in non-Bt and Bt Cry51Aa2 cotton. Graham and Stewart (2018) found increased insecticide efficacy on cotton expressing Bt Cry51Aa2 compared to non-Bt cotton. For cotton sprayed weekly with insecticides, the number of tarnished plant bugs found in drop cloth samples was reduced by 88% in Bt Cry51Aa2 cotton and 77% in non-Bt cotton when compared with plots not treated with insecticide. This finding suggests that tarnished plant bugs exposed to Bt Cry51Aa2 might be more sensitive to insecticides because they are already somewhat stressed by the Bt toxin. It might also mean that despite a similar distribution within the canopy, tarnished plant bugs exposed to the Bt toxin are moving more within the canopy, and thus, are more likely to contact insecticide residues.

In this study, we found no difference in the size of tarnished plant bug nymphs observed on Bt Cry51Aa2 and non-Bt cotton (Table 4). This finding is somewhat contradictory to a previous and more intense study (Graham and Stewart 2018) which found a significant reduction (≈ 50%) in the number of large tarnished plant bug nymphs observed. However this study was similar to Graham and Stewart (2018) in that there was little effect of Bt Cry51Aa2 on numbers of smaller nymphs. A reduction in the number of large nymphs is important because they cause more damage than smaller nymphs (Cooper and Spurgeon 2013). A reduction in the level and severity of square, flower, and boll injury was observed in Bt Cry51Aa2 cotton compared with non-Bt cotton (Tables 5 and 7), and fewer tarnished plant bugs were found in the flowers of Bt Cry51Aa2 cotton (Table 4). The protection of these fruiting structures provided by Bt Cry51Aa2 is important, especially in the bottom of the plant canopy where there is the potential for reduced insecticide efficacy (Sumner and Herzog 2000).

In our choice tests, tarnished plant bug adults avoided diet containing lyophilized Bt Cry51Aa2 leaves, and they also preferred non-Bt cotton squares over squares from Bt Cry51Aa2 plants. Egg deposition in the whole-plant cage study, in the diet pack assay, and on excised squares followed a similar trend. It is not surprising that oviposition would be lower if tarnished plant bugs avoided cotton expressing Bt Cry51Aa2. The reduction of oviposition associated with exposure to Bt Cry51Aa2 was probably related to the adult avoidance behavior rather than sublethal effects of the toxin of adult fecundity because our assays were short in duration (24 h).

No preference in diet-pack assays with first or third instar tarnished plant bugs was observed. A lack of response in first instars is not unexpected because they feed little, if at all and are not as mobile as adults. Stewart et al. (1992) found that although first instar *L. hesperus* caused feeding punctures in diet packs, they did not ingest a significant amount of diet. The host plant on which an immature plant bug develops is largely determined by the ovipositional preference of adult females. Indeed, nymphs may have less ability than adult females in discerning the quality of food sources or detecting toxins. If nymphs do have less ability to detect toxins, it could help with the efficacy of the trait, because if nymphs are not detecting Cry51Aa2 enough to elicit avoidance, they will be more likely to continue feeding on cotton expressing the toxin. A study by Graham et al. (2016) found that third instar tarnished plant bugs avoided green beans treated with field rates of various insecticides, suggesting that nymphs can have an avoidance response if the stimulus is strong enough. Because it is known that Cry51Aa2 is relatively slow (~ 6 d) to kill nymphs (Baum et al. 2012), it is possible that third instars were not exposed to enough toxin to elicit a behavioral response in 24 h, but a longer assay may have come to a different conclusion.

The efficacy of cotton varieties expressing Bt Cry51Aa2 could be reduced in large fields if efficacy is partly based on avoidance and alternative food sources are less readily available. For example, small plot research showed significant reductions of tarnished plant bugs in nectariless cotton varieties compared to nectaried cotton varieties (Meredith et al. 1973, Adjei-Maafo and Wilson 1983, Bailey et al. 1984). However, Scott et al. (1988) reported that no reduction in tarnished plant bug populations would be seen in nectariless cotton varieties at a field size of 31 ha. Further research needs to be conducted to determine how deployment of Bt Cry51Aa2 cotton in large fields might affect populations of thrips and tarnished plant bug. The yield increase reported in this paper (e.g., 57% yield increase above non-Bt plots) was almost certainly the result of differences in tarnished plant bug injury because thrips injury was managed with insecticides and did not reach levels expected to cause yield loss. Similar numbers of tarnished plant bugs were found on Bt Cry51Aa2 and on non-Bt cotton in drop cloth samples, suggesting that the mode of action of Bt Cry51Aa2 on tarnished plant bug extends beyond mere avoidance. This suggestion is supported by our data showing substantially higher yield and an obvious reduction in tarnished plant bug injury to fruiting structures despite similar numbers of tarnished plant bugs on Bt Cry51Aa2 and non-Bt cotton. While Bt Cry51Aa2 cotton appears to provide some efficacy against thrips and tarnished plant bugs, previous research indicates that supplemental applications of insecticide will still be needed at times to control tarnished plant bugs (Graham and Stewart 2018).

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Appendix II

Table 2. Mean number (SEM) of tobacco, soybean, and other adult thrips per ten plants collected from a choice test between Bt Cry51Aa2 and non-Bt seedling cotton averaged across years (2017 and 2018) in Tennessee.

	Bt	Non-Bt
Tobacco Thrips	2.31 (0.54) b	6.13 (0.59) a
Soybean Thrips	0.56 (0.27) a	0.88 (0.30) a
Other Thrips	0.88 (0.24) a	1.44 (0.42) a
Total Thrips	3.81 (0.67) b	8.44 (0.82) a

Means, within rows, followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

Table 3. Total number (percentage) of tobacco, soybean, and other adult thrips collected from field tests on Bt Cry51Aa2 and non-Bt cotton that was not treated with insecticides for thrips. Data was averaged across years (2016 and 2017) and locations (Jackson, TN and Milan, TN).[1][2]

	Tobacco Thrips	Soybean Thrips	Other Thrips	Total No. Collected
Bt	292 (76.2%)	32 (8.4%)	59 (15.4%)	383
Non-Bt	629 (76.2%)	76 (9.2%)	120 (14.5%)	825
Total	921 (76.2%)	108 (8.9%)	179 (14.8%)	1208

[1] Data from Graham and Stewart (2018) showing distribution of thrips species between Bt Cry51Aa2 and non-Bt cotton. $Chi^2 = 0.84$.

[2] Means, within columns, followed by a common letter are not significantly different (Fisher's Protected LSD, $\alpha = 0.05$).

Table 4. Impact of Bt Cry51Aa2 on mean number (SEM) of tarnished plant bug nymphs, by size of nymphs, visually observed per plant on Bt Cry51Aa2 and non-Bt cotton in Jackson, TN.

Life Stage	Non-Bt	Bt	*F*	df	*P*
Small Nymphs[1]	0.45 (0.05) a	0.45 (0.05) a	0.01	1, 197	0.9302
Medium Nymphs[2]	0.27 (0.05) a	0.27 (0.05) a	0.01	1, 182.3	0.9129
Large Nymphs[3]	0.34 (0.05) a	0.28 (0.05) a	0.71	1, 176.1	0.4014

Means, within rows, followed by a common letter are not different (Fisher's Protected LSD, α=0.05).
Small nymphs= 1st and 2nd instars
[2]Medium nymphs= 3rd and 4th instars
[3]Large nymphs= 5th instars

Table 5. Impact of Bt Cry51Aa2 on mean number (SEM) of tarnished plant bugs (TPB) visually observed on squares and flowers of Bt Cry51Aa2 and non-Bt cotton in Jackson, TN.

Squares	Non-Bt	Bt	*F*	df	*P*
Adults	0.25 (0.25) a	0.50 (0.50) a	0.20	1, 6	0.6704
Nymphs	5.00 (1.08) a	2.50 (0.87) a	3.25	1, 6	0.1210
Total TPB	5.25 (1.31) a	3.00 (0.91) a	1.98	1, 6	0.2095
Flowers					
TPB Adults	3.13 (0.61) a	0.13 (0.13) b	12.70	1, 14	0.003
TPB Nymphs	3.50 (0.57) a	1.13 (0.35) b	23.17	1, 14	<0.001
Total TPB	6.63 (0.91) a	1.25 (0.31) b	31.94	1, 14	<0.001

Means, within rows, followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

Table 6. Impact of Bt Cry51Aa2 on average number (SEM) of injured flowers from tarnished plant bug feeding observed in Bt Cry51Aa2 and non-Bt cotton in Jackson, TN.

	Non-Bt	Bt
No Injury	17.00 (1.29) a	7.50 (1.09) b
Low Injury	11.13 (1.13) a	7.50 (1.18) b
High Injury	6.25 (0.56) a	0.38 (0.26) b
Total Injured	17.38 (1.16) a	7.88 (1.27) b

Means, within rows, followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

Table 7. Average number (SEM) of tarnished plant bugs (TPB) and stink bugs from various stink bug species found per 3.02 m row of Bt Cry51Aa2 and non-Bt cotton in Jackson, TN.

Insects	Non-Bt	Bt
TPB Adults	0.88 (0.39) a	0.13 (0.13) b
TPB Nymphs	17.38 (3.67) a	20.25 (4.95) a
TPB Total	18.25 (3.43) a	20.38 (4.89) a
Total Stink Bugs	1.25 (0.49) a	1.37 (0.71) a

Means followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

Table 8. Impact of Cry51Aa2 on mean (SEM) ratings of outer boll stains, boll stain severity[1], inner boll stains, warts, and percent lint staining of cotton bolls sampled from Bt Cry51Aa2 and non-Bt plots related to damage from tarnished plant bugs in Jackson, TN.

Boll Injury	Non-Bt	Bt	*F*	df	*P*
Outer Stains	17.28 (1.62) a	9.78 (0.88) b	17.65	1, 187	<0.0001
Boll Stain Severity[1]	1.38 (0.06) a	1.14(0.03) b	11.63	1, 186	0.0008
Inner Stains	1.68 (0.24) a	0.74 (0.19) b	10.30	1, 192	0.0016
Warts	3.28 (0.34) a	2.13 (0.27) b	8.12	1, 193	0.0048
% Lint Staining	19.42 (2.59) a	9.91 (1.87) b	8.81	1, 198	0.0034

Means followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

[1]Boll staining severity ranged from 0 – 3 (0= no staining).

A — Bt Cry51Aa2

Node	T	I	II	III	IV	Node Sum	Vegetative
Pos. Sums		34	39	15	3		4
	15	4				4	
	14	1				1	
	13	5	0			5	
	12	4	4	0	1	9	
	11	8	9	0	0	17	
	10	4	10	3	0	17	
	9	3	6	0	1	10	
	8	2	6	6	0	14	
	7	0	2	4	0	6	
	6	0	1	1	1	3	
	5	1	0	1	0	2	
	4	0	1	0	0	1	
	3	1	0	0	0	1	

Position

B — Non-Bt

Node	T	I	II	III	Node Sum	Vegetative
Pos. Sums		45	33	12	3	4
	15	2			2	
	14	3			3	
	13	5	2		7	
	12	9	2		11	
	11	10	6		16	
	10	7	11	1	19	
	9	6	4	3	13	
	8	0	2	6	8	
	7	2	5	2	9	
	6	0	1	0	2	3
	5	0	0	0	1	1
	4	1	0	0	0	1
	3	0	0	0	0	0

Position

Figure 7. Total number of tarnished plant bugs observed by node and fruiting position for A) Bt Cry51Aa2 and B) Non-Bt cotton. Position = any reproductive structure found at a given position; vegetative = leaves.

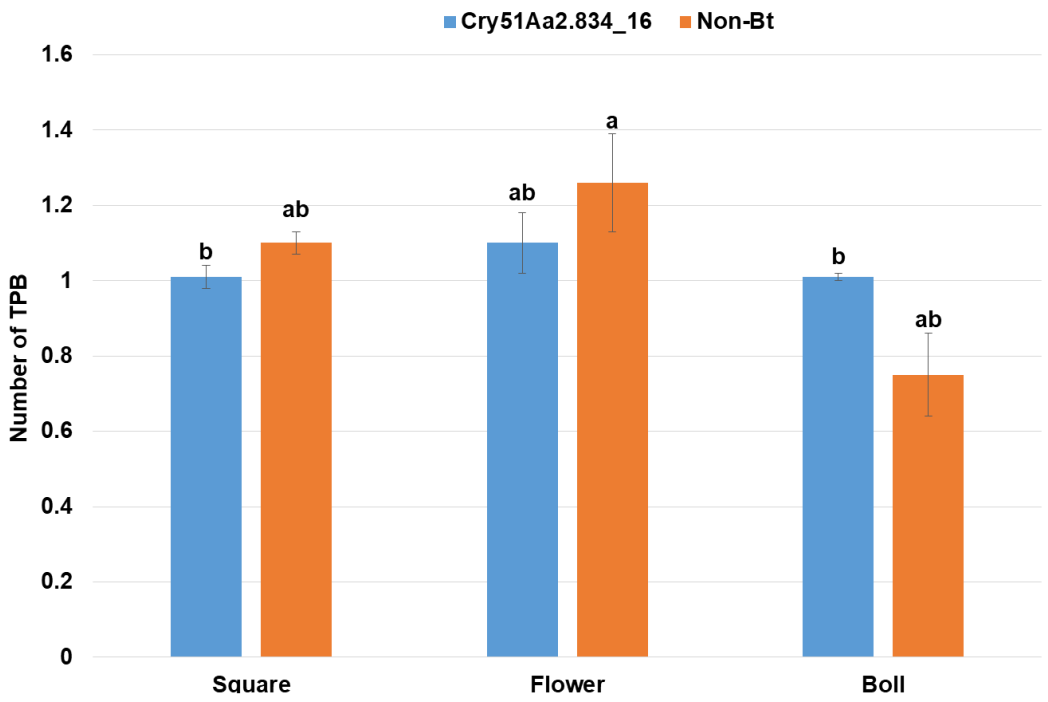

Figure 8. Influence of Bt Cry51Aa2 and non-bt cotton on the distribution of tarnished plant bug on cotton reproductive structures when not sprayed with insecticides. Error bars represent 95% confidence intervals. Common letters above bars indicate treatments are not different (Fisher's protected LSD, α = 0.05).

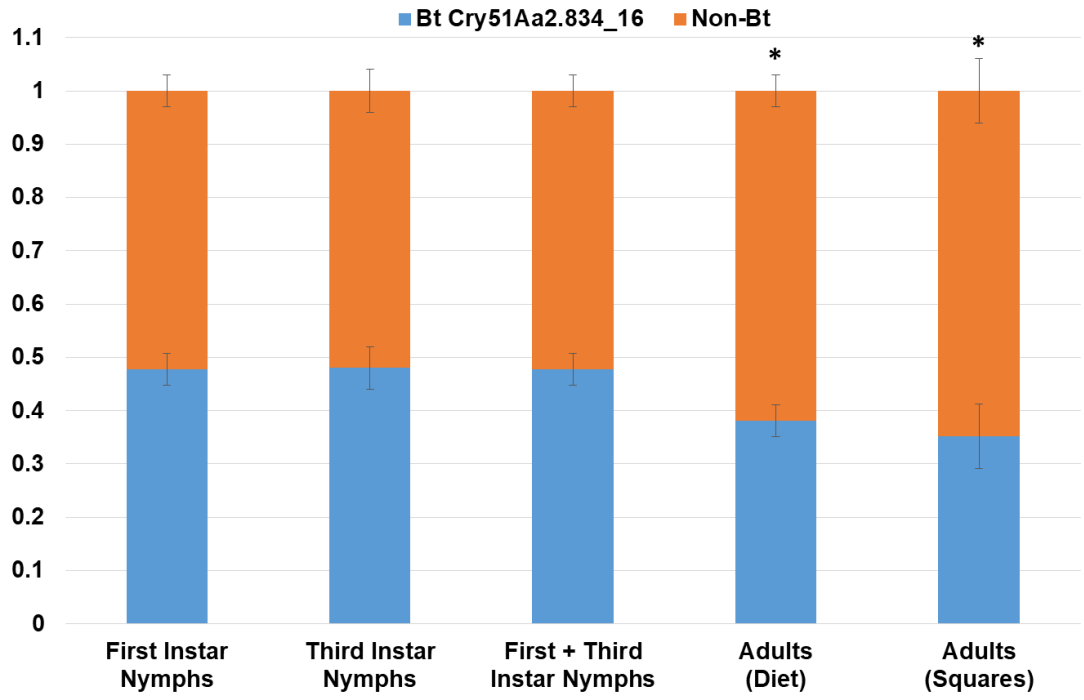

Figure 9. Overall proportion of tarnished plant bugs observed on diet packs containing lyophilized Bt Cry51Aa2 and non-Bt leaf tissue. Error bars represent 95% confidence intervals. An asterisks above bars indicate treatments are different (Fisher's protected LSD, α = 0.05).

CHAPTER III

THE USE OF CANOPEO TO RATE SEEDLING COTTON HEALTH IN SMALL-PLOT RESEARCH

Abstract

Field experiments were conducted in 2017 and 2018 in west Tennessee to determine if Canopeo (Oklahoma State University, Stillwater, OK), an image analysis tool available as a smart phone app, can be used to supplement current methods to estimate the health of seedling cotton, *Gossypium hirsutum* L., in small-plot research tests. Six tests, showing a range of cotton seedling health, were used in this analysis. Cotton seedlings in replicated small-plot tests were visually rated for vigor and thrips (Thysanoptera: Thripidae) injury. Above-ground biomass samples were collected in three of the tests. Additionally, a photograph of the center two rows of each plot was taken and analyzed using Canopeo. Strong correlations were observed for Canopeo and biomass, Canopeo and vigor, and thrips injury ratings and biomass. These data suggest that the Canopeo would be a useful and non-destructive tool to objectively assess treatment effects on plant health in small-plot cotton research trials.

Keywords: Cotton, Seedling Health, Thrips, Canopeo

Introduction

Obtaining a uniform, vigorous stand of cotton, *Gossypium hirsutum* L., is among the most important aspects of cotton production. Seed germination and seedling vigor are largely determined by the physical and chemical characteristics of the seed (Snider and Oosterhuis 2015) and are closely associated with seed density and size (Krieg and Bartee 1974), seed filling (Ferguson and Turner 1971), and the lipid content of the cotton seed (Bartee and Krieg 1974). Germination and early season vigor are also dependent on conditions at planting. However, after germination several factors can affect seedling health, including insect pests and seedling diseases.

Insect pests, such as thrips (Thysanoptera: Thripidae), can lead to reduced cotton stand, stunted growth, and delayed fruiting (Layton and Reed 2002). When thrips populations are high,

they can injure plants by feeding on the contents of epidermal cells, leading to the removal of cell contents and a silvery appearance of the injured cells (Telford and Hopkins 1957, Reed and Reinecke 1990). This injury can lead to distortion and tearing of new leaves as well as cause leaf margins to curl upwards and inwards towards the mainstem (Telford and Hopkins 1957). Often, high infestations of thrips in combination with poor growing conditions result in reductions of cotton plant height and leaf area (Burris et al. 1989), leading to low cotton seedling vigor and delayed maturity extending into the late growing season (Lentz and Austin 1994, Stewart et al. 2013). Vineyard et al. (2017) found that the use of an insecticide seed treatment increased both seedling vigor and above ground cotton biomass. These same authors and Copeland et al. (2017) also reported that some pre-emergence herbicides negatively affected seedling vigor and/or biomass.

Cotton seedling diseases can affect cotton germination and emergence, survival, and seedling vigor (Rothrock et al. 2012). *Pythium* spp. are the most common pathogen isolated from cotton seedlings and can lead to seed rot and pre-emergence damping off (Rothrock et al. 2012). Johnson and Doyle (1986) found a negative correlation between percentage of cotton seedlings with *Pythium* spp. and percent emergence. *Pythium* can also affect the seedling stem, leading to post-emergence damping off, plant stunting, and chlorosis. Plants exhibiting post-emergence damping off are typically stunted and a lighter green color than normal, leading to wilting and lesions near the soil line. As the lesions progress they become darker in color until the area develops into a black “wire stem” and eventually die, leaving an uneven stand (Allen 2011). *Rhizoctonia solani* is also an important pathogen of seedling cotton. This soil-borne pathogen can lead to the death of seedling plants due to postemergence damping-off or “shore shin”

(Newman 2001). Vineyard et al. (2017) also reported an increase of cotton seedling vigor and above ground plant biomass with the use of a fungicide seed treatment.

Currently, the most common way to evaluate cotton seedling health is by subjective visual ratings, such as seedling vigor ratings or thrips injury ratings. Vigor ratings can be based on a visual rating of the whole plot, by determining the number of true leaves per row foot, or by taking plant height counts. Thrips injury ratings are often made by rating an entire plot on a 0 – 5 or 1 – 5 scale, where 0 (or 1) is no injury and 5 is no living plants in the plot. Although useful, visual ratings are subjective and relative, and thus, are subject to bias. Cotton seedling health can also be accessed by measuring plant biomass by cutting and weighing plants. Although objective, a destructive sampling method is often not compatible with small-plot research. The use of remote sensing technology is a newer method used estimate to cotton stands and plant health. The normalized difference vegetation index (NDVI) (Tucker 1979) is the most common vegetative index used for measuring plant health (Plant et al. 2001). Remote sensing equipment can be attached to ground rigs or unmanned aerial vehicles in order to cover large areas of ground efficiently (Sui et al. 2017). Recently, an image analysis tool, Canopeo, has been developed at Oklahoma State University in the Matlab programming language (Mathwork, Inc., Natick, MA) using color values in the red-green-blue system (Patrignani and Oschner 2015). This software quantifies fractional green canopy cover (FGCC) to estimate plant canopy development. When compared to two other software packages used to analyze FGCC, Canopeo was faster than both and comparable in accuracy (Patrignani and Ochsner 2015). Canopeo has been developed as an application for mobile devices using iOS and Android processing systems. The ease of which this application can be used by researchers makes it an intriguing way to rate treatment effects in small-plot cotton tests related to thrips injury or seedling disease. The intent

of this study was to determine if Canopeo can be used as an objective sampling method compared with subjective, visual thrips assessments of thrips injury, or plant health in general, in cotton research trials.

Materials and Methods

Tests were done in 2017 and 2018 at the West Tennessee Research and Education Center (Jackson, TN) and at the Research and Education Center at Milan (Milan, TN). A total of six tests were selected to evaluate the relationship between various measures of cotton seedling plant health including visual estimates of vigor and thrips injury, above ground biomass, and measurements using Canopeo. In 2017, identical tests were conducted at Jackson (test 1) and Milan (test 2), and tests 3 and 4 were performed at Jackson. Tests 5 and 6 were conducted at Jackson during 2018. Treatments factors in each test are listed in Table 9. Each test was arranged as a randomized complete block design with four or five replications. Individual plots were 10.7 m in length and 4 rows wide, planted no-till on 0.97 m centers. Cotton varieties varied across but not within tests. These tests were selected for Canopeo measurements because they showed a wide range of treatment effect on seedling health, primarily driven by the thrips treatment regimen.

Visual estimates of thrips injury and plant vigor were made to evaluate treatment effects on plant health. These ratings were made at the 3.5 true-leaf stage on a whole plot basis. Ratings for thrips injury were made on a 0 – 5 scale where 0 was no injury to any plant in the plot, 3 is moderate injury, and 5 is no living plants in the plot. Plant vigor rating was made on a 0 – 5 scale where 0 represents no living plants in the plot and 5 represents maximum vigor. Also, photographs of the center two rows of each plot were taken and analyzed using the automatic color threshold (ACT) image analysis tool, Canopeo. Photographs were made at the front of each

plot and taken from roughly three feet above the ground. The camera was angled so that as much of the center two rows as possible would be in the photograph without bordering rows being within the field of view. In order to reduce white pixels in the middles between rows, the 'slider' was adjusted to match the green pixels in the original photograph (Lollato et al. 2015). Within each test, the same value was used on the 'slider' (Fig. 10). Above ground cotton biomass was sampled in tests one, two, and three only. A total of five plants were collected from each plot. Plants were cut at the ground level and placed in plastic bags. The fresh weight of each sample was recorded and converted to biomass per two rows based on cotton stand counts taken in each plot.

Statistical Analysis

Data was analyzed using Spearman's correlation to see how vigor, thrips injury, and biomass ratings correlated with Canopeo. Thrips injury was reverse coded to have the same direction as the other variables. The min-max scaling method was used to rescale vigor, thrips injury, Canopeo and biomass ratings to maintain the distributional probability. The Bland-Altman plot method was used to assess the agreement of thrips injury, plant biomass, and plant vigor with Canopeo ratings (Bland and Altman 1986). The difference between two methods was regressed on the averages to detect whether there was a significant trend on bias when the magnitude of measurements increased. The significance level is 0.05 and statistical analysis was performed using SAS 9.4. (SAS Institute, Cary, NC). Although data was rescaled prior to analysis, scatterplots were made to show the relationships between the raw data points (Fig. 11 and 12).

Results and Discussion

For all variables, 95% of the data points were within the 95% limit of agreement, therefore further analysis was done (Fig. 13 and 14). Data were considered in agreement if the Bland-Altman regression analysis was not significant ($P>0.05$). As the slope of the Bland-Altman regression or the 95% limit of agreement range decreases, data agreement gets stronger. Canopeo data was in agreement with above ground plant biomass and plant vigor (Table 10). Agreement was also found for plant vigor and above ground plant biomass data. After Bland-Altman regression was done, data were analyzed using Spearman correlation. A correlation was observed between vigor and Canopeo ($r=0.67$) and biomass and Canopeo ($r=0.72$). As vigor or biomass increased, so did Canopeo ratings. A positive correlation of biomass and vigor ($r=0.56$) was also observed.

No agreement was found for thrips injury data with Canopeo, plant vigor or above ground plant biomass ratings. However, trends were observed for thrips injury and Canopeo ratings ($r=0.65$) and thrips injury with plant vigor ($r=0.69$) and a weaker correlation for thrips injury and biomass ($r=0.41$). Canopeo ratings, plant vigor, and above ground plant biomass ratings tended to decreased when thrips injury increased.

This data suggests that the image analysis tool, Canopeo, can be used as an objective method to evaluate treatment effects on cotton seedling health in small-plot research. In particular, Canopeo can be used instead of or supplemental to plant vigor ratings and above ground biomass ratings. One potential pitfall would be the presence of weedy vegetation that would interfere with Canopeo measurements. However, the use of Canopeo should help standardize evaluations across multiple tests or years, and sampling bias would likely be reduced. It is also a non-destructive way to estimate above ground plant biomass. When sampling for biomass, plants must be removed from plots, and thus, a small amount of plants are

often sampled. Human bias can play a role in determining which plants are selected. Canopeo takes into account a much larger percentage of the plot. Although the correlation was not as strong (r=0.65), the data suggests that Canopeo ratings could also be used to supplement, not replace, thrips injury ratings. Although biomass, vigor, and thrips injury are often correlated, they are not necessarily related. Biomass and vigor ratings can be compounded by factors other than thrips injury, such as herbicide injury, seedling diseases, poor cotton growing conditions, or nematodes. It also seems likely, based on our observations, that Canopeo would have utility in evaluating seedling health related to any number of factors including seedling disease, herbicide injury or varietal vigor.

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Appendix III

Table 9. List of treatments in six tests used to evaluate the use of Canopeo for cotton seedling health ratings. Insecticide = neonicotinoid seed treatment or foliar application for thrips.

Test	Treatment	Insecticide	Base Fungicide	Premium Fungicide	Bt Cry51Aa2[1]	Seeds/Ft
1, 2	1	Yes	Yes	No	Yes	4
	2	No	Yes	No	Yes	4
	3	Yes	Yes	No	No	4
	4	No	Yes	No	No	4
3	1	No	No	No	No	4
	2	No	Yes	No	No	4
	3	Yes	Yes	No	No	4
	4	Yes	Yes	No	No	4
4	1	No	Yes	No	No	4
	2	Yes	Yes	No	No	4
	3	Yes	Yes	No	No	4
	4	Yes	Yes	No	No	4
	5	Yes	Yes	No	No	4
	6	Yes	Yes	No	No	4
5	1	No	Yes	No	No	4
	2	Yes	Yes	No	No	4
	3	Yes	Yes	No	Yes	4
	4	No	Yes	No	Yes	4
6	1	No	Yes	No	No	3
	2	No	Yes	No	No	4
	3	Yes	No	No	No	3
	4	Yes	No	No	No	4
	5	Yes	Yes	No	No	3
	6	Yes	Yes	No	No	4
	7	No	No	Yes	No	3
	8	No	No	Yes	No	4
	9	Yes	No	Yes	No	3
	10	Yes	No	Yes	No	4

[1]Transgenic Bt toxin with reported suppression of thrips (Graham and Stewart 2018).

Table 10. Bland-Altman analysis for regression of Canopeo, biomass, thrips injury, and plant vigor.

Variable	Mean Bias (±SEM)[1]	P	95% Limit of Agreement Range[2]	Slope	Spearman's Correlation
Canopeo vs Injury	0.202 (0.245)	<0.001 *	0.961	0.312	0.65
Canopeo vs Biomass	-0.145 (0.165)	0.527	0.647	0.045	0.72
Canopeo vs Vigor	0.404 (0.208)	0.492	0.816	0.048	0.67
Injury vs Biomass	0.145 (0.266)	0.023*	1.042	0.332	0.41
Injury vs Vigor	0.201 (0.189)	<0.001 *	0.741	0.232	0.69
Biomass vs Vigor	0.234 (0.221)	0.196	0.866	0.126	0.56

[1]The difference of the mean between the two measurements.
[2]The difference between the upper and lower 95% limits of agreement.
*Data does not agreement in Bland-Altman analysis.

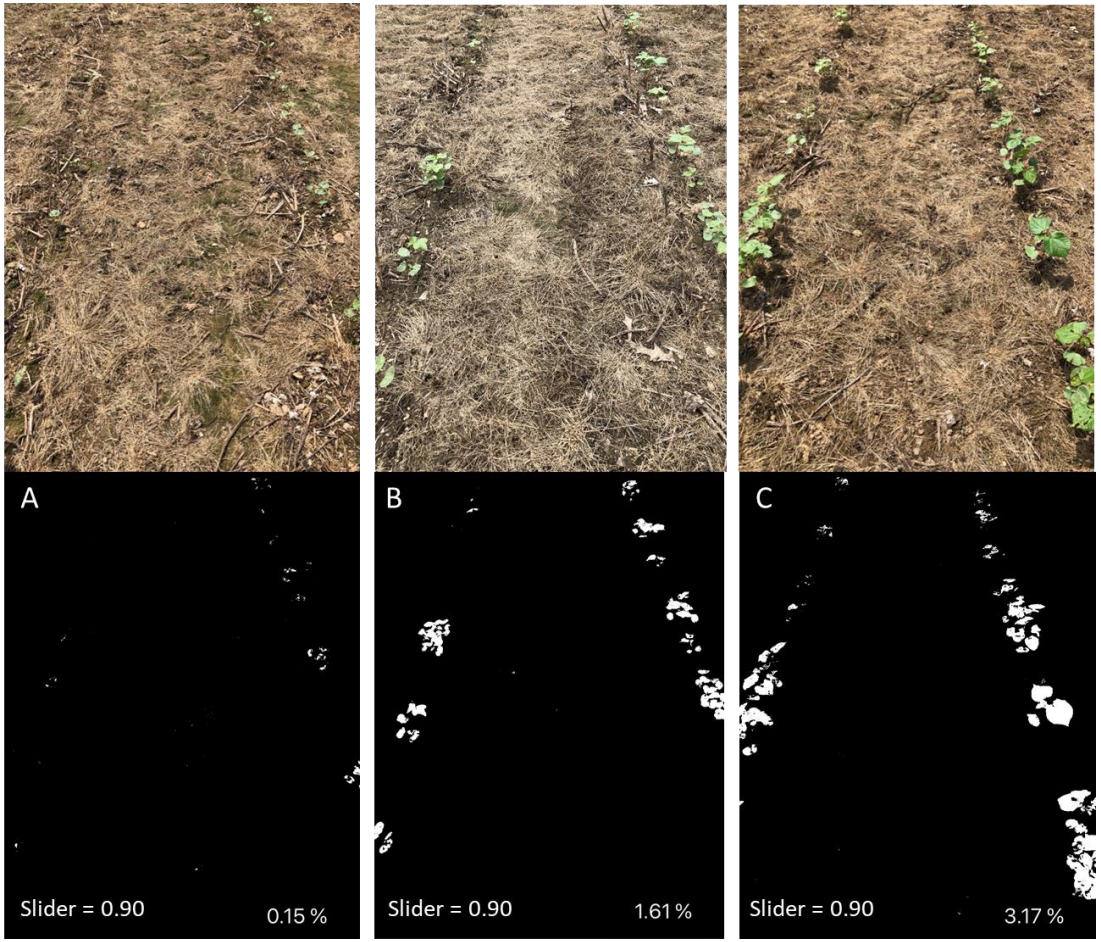

Figure 10. Example of photographs analyzed using Canopeo showing A. poor cotton seedling vigor B. moderate cotton seedling vigor C. good cotton seedling vigor.

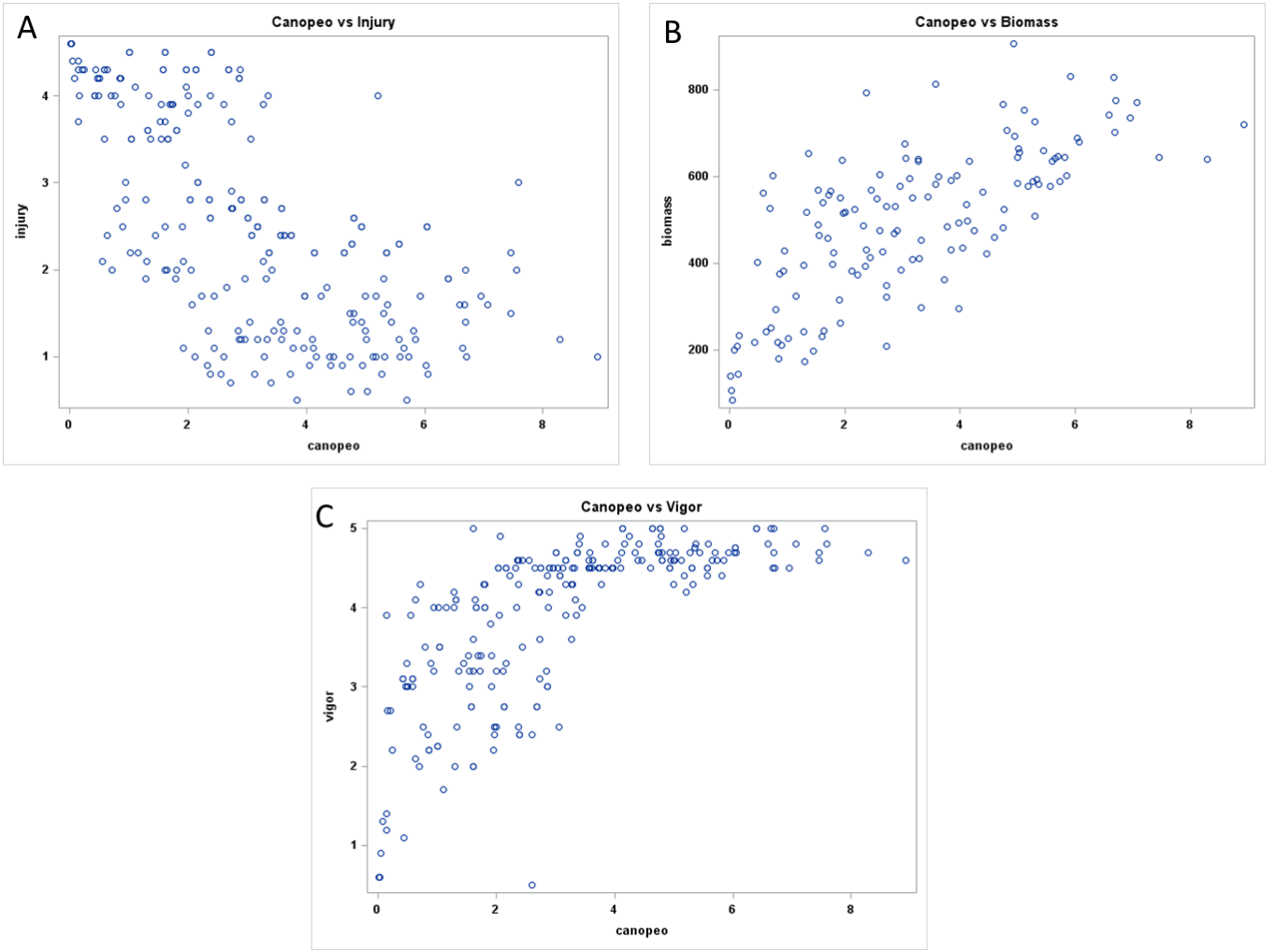

Figure 11. Scatter plot of raw data for A. Canopeo vs. thrips injury, B. Canopeo vs. above ground plant biomass, and C. Canopeo vs. plant vigor.

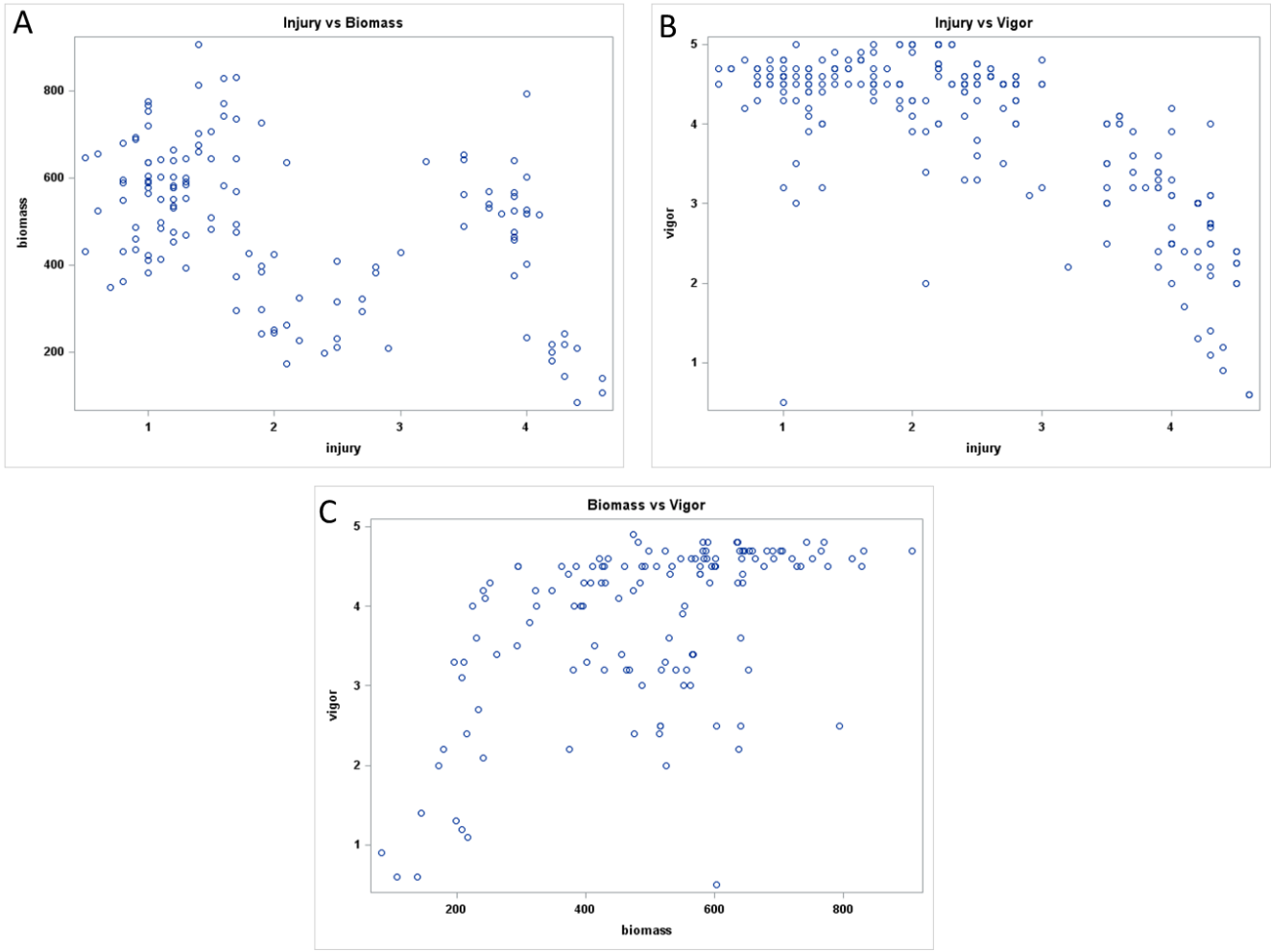

Figure 12. Scatterplot of raw data for A. thrips injury vs. above ground plant biomass, B. thrips injury vs. plant vigor, and C. above ground plant biomass vs. plant vigor.

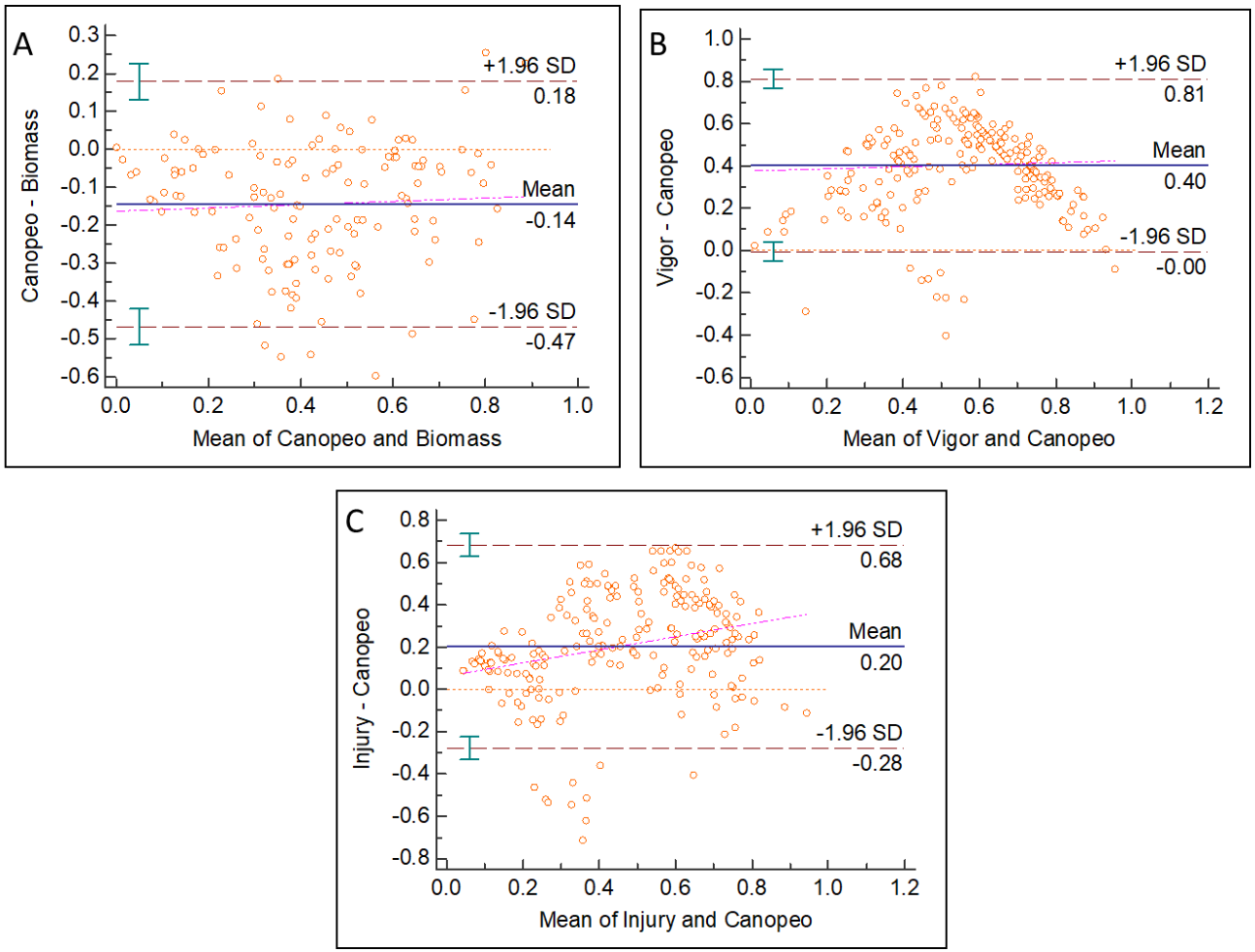

Figure 13. Bland-Altman plot showing mean bias line, zero bias line, regression line and 95% limits of agreement for A. thrips injury vs. above ground plant biomass, B. thrips injury vs. plant vigor, and C. above ground plant biomass vs plant vigor.

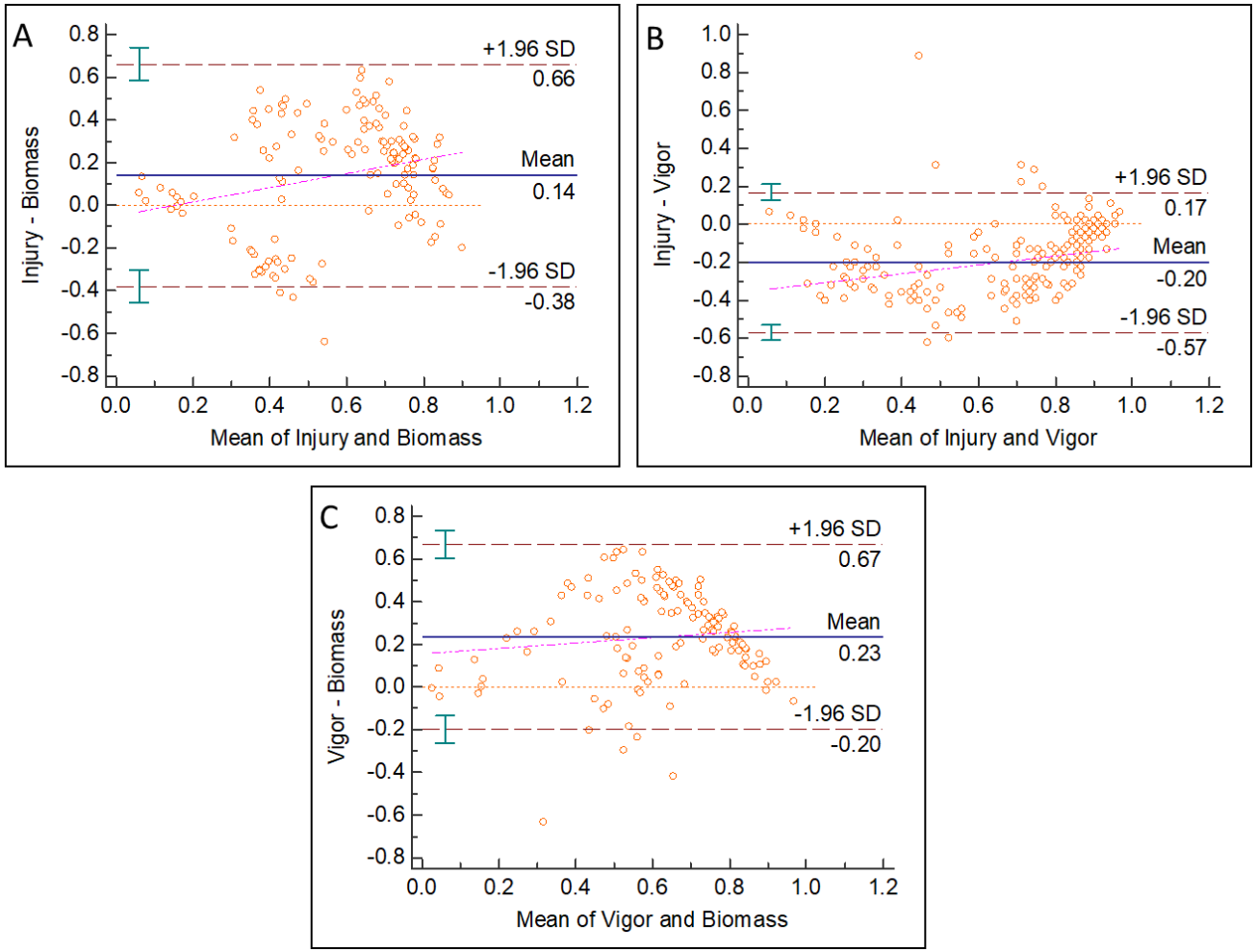

Figure 14. Bland-Altman plot showing mean bias line, zero bias line, regression line and 95% limits of agreement for A. thrips injury vs. above ground plant biomass, B. thrips injury vs. plant vigor, and C. above ground plant biomass vs. plant vigor.

CONCLUSIONS

The main objective of my research was to evaluate the effects of Bt Cry51Aa2.834_16 on thrips and tarnished plant bug management in cotton. The first component of my project was a field trial designed to observe the effects of the Bt trait for the primary target pests (thrips and tarnished plant bug) of cotton in an insecticide-free system and a system including insecticide management. Results from this study showed that Bt Cry51Aa2 provides as good or better protection from thrips injury compared to the current best alternative strategy (insecticide seed treatment [IST] + foliar application). However, when an IST + a foliar application was applied to the Bt trait, even greater protection from thrips was observed over the Bt trait alone. The Bt trait also had significant impacts on tarnished plant bug. Regardless of insecticide strategy, there was significantly higher square retention, fewer tarnished plant bug adults prior to bloom, and fewer total tarnished plant bug nymphs, especially large nymphs, during bloom in Bt plots compared with non-Bt plots. Yield (kg seed-cotton/ ha) was higher in Bt plots than non-Bt plots when no insecticides were used for tarnished plant bug or when weekly applications were made. When plots were managed for tarnished plant bug using current thresholds, no difference in yield was observed, however an average of 1.2 fewer insecticide applications were required for Bt plots over non-Bt. Based on these results, Bt Cry51Aa2.834_16 is expected to be a valuable asset to an overall cotton insect pest management program.

The second component of my research focused on the behavioral responses of thrips and tarnished plant when exposed to Bt Cry51Aa2.834_16. Adult thrips avoided Bt cotton when given the choice of non-Bt cotton in field tests. In greenhouse tests, a ratio of ≈ 2:1 adult thrips and thrips eggs were found in non-Bt cotton over Bt cotton. A Chi^2 analysis of adult thrips species distribution showed no effect of the Bt trait on the species observed. In a field test of

cotton not sprayed with insecticides for tarnished plant bug, no difference in the distribution of tarnished plant bug was found between Bt and non-Bt cotton; however, a difference of injury was observed. More square, flower and boll injury was found in non-Bt plots. The differences in injury were also apparent in yield as Bt cotton had higher yields than non-Bt. In laboratory studies using tarnished plant bug diet incorporated with leaf tissue of Bt and non-Bt plants and a study using excised squares of each, adult tarnished plant bugs avoided the Bt trait. No avoidance was observed for first or third instars. Although there was a difference in the number of tarnished plant bug eggs laid in diet packs and excised squares, no difference was observed for the number of eggs found in a whole plant caging study between Bt and non-Bt cotton. Thus, the difference in egg lay in the laboratory studies were likely related to the avoidance behavior of the adults rather than sub-lethal effects of the toxin. Overall, avoidance appears to be an important mode of action of the Bt toxin. Avoidance behavior could have potential implications on field control, as results may vary if this technology is adopted in large fields. A shift to larger fields could also affect the risk of insect resistance and how management programs are implemented.

The last component of my research was to determine if the image analysis tool Canopeo could be used to supplement current methods to estimate cotton seedling health. These data suggested that Canopeo can be used instead of or supplemental to both plant vigor ratings and above ground biomass ratings. Canopeo also appears to have utility in supplementing, not replacing thrips injury rating. Based on our observations, we concluded that Canopeo would have utility in evaluating seedling health related to any number of factors including seedling disease, herbicide injury or varietal vigor.

APPENDIX: SUPPLEMENTARY TABLES

Table S1. Average total number (Mean ± SEM) of thrips per five plants, average thrips injury rating, average above ground biomass per five plants, and average vigor ratings for Bt Cry51Aa2 and non-Bt cotton, with and without an insecticide seed treatment (IST) averaged across two years and two locations.

	Bt		Non-Bt	
	IST	No IST	IST	No IST
Thrips	7.83 (0.66)c	22.44 (1.41)b	20.15 (1.62)b	78.10 (4.95)a
Thrips injury [1]	0.64 (0.03)d	0.88 (0.03)c	1.20 (0.05)b	3.02 (0.08)a
Biomass (g)	3.62 (0.10)a	3.13 (0.08)b	3.46 (0.10)a	2.99 (0.08)b
Vigor [2]	4.45 (0.06)a	4.18 (0.18)b	4.51 (0.08)a	2.9 (0.10)c

Means followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

[1] Visual rating of thrips injury on a 0 – 5 scale where 0 represents no injury to any plant in a plot.

[2] Visual rating of plant vigor on a 0 – 5 scale where 0 indicates no living plants in a plot.

Table S2. Total number of foliar insecticide applications made to manage tarnished plant bugs in Bt Cry51Aa2 and non-Bt cotton managed using a threshold approach, with and without an insecticide seed treatment (IST).

Location	2016				2017			
	Bt		Non-Bt		Bt		Non-Bt	
	IST	No IST	IST	No IST	IST	No IST	IST	No IST
Jackson	4	3	6	7	2	2	3	2
Milan	4	4	5	5	1	1	2	1

Table S3. Average total number of tarnished plant bug nymphs found per 3.02 m row (Mean ± SEM) of blooming Bt Cry51Aa2 and non-Bt cotton with and without an insecticide seed treatment (IST), and managed for tarnished plant bug with different spray regimens[1] averaged across two years and two locations.

	IST			No IST		
	None	Threshold	Auto	None	Threshold	Auto
Total Nymphs	16.41 (0.95)b	6.27 (0.58)c	3.23 (0.42)de	19.99 (1.06)a	4.89 (0.47)cd	2.98 (0.40)d
	Bt			Non-Bt		
	None	Threshold	Auto	None	Threshold	Auto
Total Nymphs[2]	16.05 (1.05)b	5.07 (0.54)c	1.88 (0.27)d	20.36 (1.03)a	6.09 (0.50)c	4.66 (0.51)c
Large Nymphs	2.64 (0.23)b	0.32 (0.05)de	0.03 (0.02)e	5.31 (0.12)a	1.05 (0.33)c	0.78 (0.12)cd

Means followed by a common letter are not significantly different (Fisher's Protected LSD, α=0.05).

[1] None = not treated for TPB, Threshold = treated based on recommended thresholds, Automatic = treated weekly for TPB.

[2] Interaction of trait and spray regimen for total nymphs approached significance (P=0.0591).

Table S4. Average total kilograms of seed-cotton per hectare (Mean ± SEM) for Bt Cry51Aa2 and non-Bt cotton treat with and without an insecticide seed treatment (IST) averaged across two years and two locations.

IST		No IST	
Bt	Non-Bt	Bt	Non-Bt
3,802 (138.35)a	3,684 (161.52)a	3,763 (138.58)a	3,362 (149.38)b

Means followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

Table S5. Average total kilograms of seed-cotton per hectare (Mean ± SEM) for Bt Cry51Aa2 and non-Bt cotton managed for tarnished plant bug with different spray regimens1 averaged across two years and two locations.

Bt			Non-Bt		
None	Threshold	Auto	None	Threshold	Auto
3,273	3,884	4,190	2,787	3,777	4,006
(158.51)d	(152.94)bc	(157.07)a	(130.33)e	(174.81)c	(194.78)ab

Means followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

[1] None = not treated for TPB, Threshold = treated based on recommended thresholds, Auto = treated weekly for TPB.

Table S6. Influence of Bt Cry51Aa2 and non-Bt cotton managed for tarnished using different spray regimens[1] on average cotton fiber quality indices (Mean ± SEM) averaged across two years and two locations.

	Bt			Non-Bt		
Quality Index	None	Threshold	Auto	None	Threshold	Auto
Strength	32.62 (0.32)c	32.89 (0.24)bc	32.55 (0.34)c	34.35 (0.31)a	33.11 (0.25)bc	33.48 (0.37)b
Yellowness	7.57 (0.06)ab	7.28 (0.06)abc	6.87 (0.40)bc	7.88 (0.07)a	6.66 (0.53)c	7.49 (0.07)ab
Micronaire	4.73 (0.07)a	4.39 (0.07)a	4.51 (0.06)a	4.79 (0.07)a	4.4 (0.06)8a	4.66 (0.06)a
Reflectance	72.11 (0.30)a	73.04 (0.22)a	72.89 (0.33)a	70.90 (0.35)a	72.19 (0.29)a	72.24 (0.37)a

Means followed by a common letter are not different (Fisher's Protected LSD, α=0.05).

[1]Unsprayed = not treated for TPB, Threshold = treated based on recommended thresholds, Automatic = treated weekly for TPB.

VITA

Scott Hester Graham was born in Plano, TX and grew up in Grenada, MS. He discovered his love for agriculture while traveling to Mississippi State football games with his great uncle, Ed Hester, and his father, Chip Graham. On these trips he would listen to his dad and Uncle Ed discuss the farming world. Scott took his interest in agriculture to Mississippi State University, where he earned a B.S. in Agronomy with a concentration in Integrated Pest Management. While earning his B.S., Scott worked as an undergraduate student worker for Mississippi State Extension Entomologist, Dr. Angus Catchot. Working for Dr. Catchot showed Scott the exciting opportunities in applied research and extension entomology. He then earned a M.S. in Entomology from Mississippi State under the direction of Dr. Catchot and Dr. Jeff Gore in 2016. After receiving his M.S., he joined the Department of Entomology and Plant Pathology at The University of Tennessee, Knoxville to pursue a PhD in Entomology under Dr. Scott D. Stewart. Scott earned his PhD in December of 2019.